The molecular determinants of methicillin-resistance and virulence of Staphylococcus aureus : a cross-border study in Europe

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A CROSS-BORDER STUDY IN EUROPE

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For those I love

I'm always living alone
An ancient cave is my home
See the look in my eyes
They think I'm a mysterious man
Cause people do all they can
To avoid evil eyes
(*Kayak – Merlin*)

CONTENTS

ABBREVIATIONS

APC	Antigen-Presenting Cell (APC)
bp	base pair
CA-MRSA	Community-Acquired Methicillin-Resistant *Staphylococcus aureus*
CC	Clonal Complex
ccr	cassette chromosome recombinase
CFU	Colony Forming Unit
CNS	Coagulase-Negative Staphylococci
dNTP	deoxyribonucleotide triphosphate
EMR	Euregio Meuse-Rhine
ET	Exfoliative Toxin
EU	European Union
HA-MRSA	Hospital-Acquired Methicillin-Resistant *Staphylococcus aureus*
MHC	Major Histocompatibility Complex
MIC	Minimal Inhibitory Concentration
MLST	Multilocus Sequence Typing
MRCNS	Methicillin-Resistant Coagulase-Negative Staphylococci
MRSA	Methicillin-Resistant *Staphylococcus aureus*
MSSA	Methicillin-Sensitive *Staphylococcus aureus*
NCCLS	National Committee for Clinical Laboratory Standards
NT	Not Typeable
PBP	Penicillin Binding Protein
PCR	Polymerase Chain Reaction
PFGE	Pulsed-Field Gel Electrophoresis
PVL	Panton-Valentine leukocidin
SAg	Superantigen
SCC*mec*	Staphylococcal Cassette Chromosome *mec*
SD	Standard Deviation
SE	Staphylococcal Enterotoxin
SLV	Single Locus Variant
ST	Sequence Type
TAE	Tris-Acetate-EDTA
TST	Toxic Shock Syndrome
TSST-1	Toxic Shock Syndrome Toxin-1
UPGMA	Unweighted Pair Group Matching Analysis
WG	Wegener's Granulomatosis

CHAPTER 1

AIMS OF THE STUDY & OUTLINE OF THE THESIS

AIMS OF THE STUDY

Since its discovery in the 1880s, *Staphylococcus aureus* has emerged as a potential pathogenic Gram-positive bacterium that can cause various diseases, ranging from skin infections to post-operative wound infections. Shortly after the introduction of methicillin in 1959, methicillin-resistant *S. aureus* (MRSA) strains have been isolated in the United Kingdom. Subsequently, several MRSA clones, which differ in their genetic background, disseminated worldwide. The pathogenicity of *S. aureus* can be attributed to the production of virulence factors, such as Panton-Valentine leukocidin (PVL) and superantigens (SAgs), like Toxin Shock Syndrome Toxin-1 (TSST-1).

Any strategies to contain the dissemination of MRSA, either in the community, the hospital, at national or international level, require knowledge about the MRSA clones that are disseminated. Cross-border patient mobility and free access to health care facilities within the European Union (EU) in general, and the border regions in particular, are important issues for patients, doctors, hospitals, sickness funds and the health care insurance companies. Cross-border transfer of patients may have an important impact on the dissemination and prevalence of MRSA, in particular in cases where patients are transferred from countries with a relatively high prevalence to a country with a low prevalence.

The aims of this study were the molecular characterisation of *S. aureus* strains from several European countries (Belgium, Croatia, Germany and The Netherlands) with respect to the resistance to methicillin and the presence of the virulence factors PVL and TSST-1, and to test the usefulness of ciprofloxacin resistance as a predictive marker for MRSA in The Netherlands.

OUTLINE OF THE THESIS

This thesis describes the characterisation of MRSA isolates from the Euregio Meuse-Rhine (EMR), the border region of Belgium, Germany and The Netherlands, and Croatia using several typing techniques, such as Pulsed-Field Gel Electrophoresis (PFGE), SCC*mec* typing and Multilocus Sequence Typing (MLST). Furthermore, the presence of the virulence factors PVL and TSST-1 was investigated. In **chapter 2** a review of the literature is presented. This review includes the construction of Staphylococcal Cassette Chromosome *mec* (SCC*mec*), the molecular evolution of both hospital-acquired (HA-MRSA) and community-acquired MRSA (CA-MRSA), the biology of virulence factors, such as PVL and TSST-1, the worldwide prevalence of HA-MRSA and CA-MRSA, and the risk factors associated with colonisation of HA-MRSA and CA-

MRSA. Since CA-MRSA isolates often harbour PVL, a real-time PCR method was developed for the detection of PVL, using clinical isolates of *S. aureus* strains, without prior DNA isolation. Furthermore, the specificity, reproducibility, and detection limit of this assay were investigated and a study was performed to investigate the prevalence of PVL among both community-acquired (CA) and hospital-acquired (HA) *S. aureus* bloodstream isolates from The Netherlands (**Chapter 3**). This real-time PCR assay was used to investigate the prevalence of PVL in the EMR. Furthermore, the clonal dissemination of MRSA isolates was investigated using PFGE. SCC*mec* typing and MLST were used to investigate which MRSA clones are present in the EMR. Finally, the relation between the antibiotic susceptibility pattern and the SCC*mec* type was investigated (**Chapter 4**). Since TSST-1 is another important *S. aureus* virulence factor, another real-time PCR was developed for the *tst* gene, encoding TSST-1. The specificity, reproducibility, and detection limit were evaluated. Furthermore, the prevalence of the *tst* gene in CA and HA *S. aureus* strains, and isolates from Wegener's Granulomatosis patients from The Netherlands was investigated. Furthermore, the clonal relationship between the *tst*-positive strains was investigated with PFGE (**Chapter 5**). This real-time PCR, together with the real-time PCR assay for PVL, was used to investigate the prevalence of PVL and TSST-1 among Croatian MRSA bloodstream isolates. Furthermore, these strains were characterised using the antibiotic susceptibility pattern and PFGE. SCC*mec* typing and MLST were used to investigate which MRSA clones are present in Croatia (**Chapter 6**). Since most of the MRSA strains studied were resistant to ciprofloxacin, a study was performed to investigate if ciprofloxacin resistance of *S. aureus* is a biological marker for MRSA in The Netherlands. Finally, the prevalence of the high-level mupirocin gene *ileS-2* was investigated with PCR among the *S. aureus* isolates from The Netherlands (**Chapter 7**). At the end, a general discussion and summary of the study is given (**Chapter 8**).

CHAPTER 2

The molecular evolution of methicillin-resistant *Staphylococcus aureus*

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ABSTRACT

Staphylococcus aureus **is a potential pathogenic bacterium that can cause various diseases.** ***S. aureus*** **has a strong adaptive power to antibiotics. Since the introduction of methicillin in 1959, MRSA strains have been isolated, and several hospital-acquired MRSA (HA-MRSA) clones have disseminated worldwide. The prevalence of HA-MRSA ranges from 0.6% in The Netherlands to 66.8% in Japan. Resistance to methicillin is caused by the** ***mec*****A gene, which is situated on a mobile genetic element, the Staphylococcal Cassette Chromosome** ***mec*** **(SCC*mec*). To date, five different SCC*mec* types (I to V) have been distinguished. All these SCC*mec* elements carry genes encoding for resistance to β-lactam antibiotics as well as genes for the regulation of expression of** ***mec*****A. Additionally, SCC*mec* types II and III carry non-β-lactam antibiotic resistance genes on integrated plasmids and transposons. SCC*mec* have been acquired at least twenty times by different lineages of** ***S. aureus*****. Although most MRSA strains are hospital-acquired, community-acquired strains (CA-MRSA) have also been reported recently. Worldwide, the prevalence of CA-MRSA is 0.5%. Several risk factors have been identified for colonisation with CA-MRSA and HA-MRSA.** ***S. aureus*** **can produce several virulence factors, such as Panton-Valentine leukocidin (PVL), which, together with SCC*mec* type IV, is suggested to be a marker for CA-MRSA. Furthermore,** ***S. aureus*** **can produce several superantigens (SAgs), such as staphylococcal endotoxins (SEs) and toxic shock syndrome toxin-1 (TSST-1), causing several diseases, like food poisoning and toxin shock syndrome (TSS).**

INTRODUCTION

Since its discovery during the 1880s, *Staphylococcus aureus* has emerged as a potential pathogenic Gram-positive bacterium that can cause various diseases, ranging from minor infections of the skin to post-operative wound infections, bacteraemia, infections associated with foreign bodies and necrotising pneumonia. Until the introduction of penicillin, the mortality rate of patients infected with *S. aureus* was about 80%. In the early 1940s, *S. aureus* infections were treated with penicillin, but in 1942 the first strains resistant to this antibiotic were isolated, first in hospitals, and later in the community. Since 1960 around 80% of all *S. aureus* strains were resistant to penicillin. These resistant strains produced a plasmid-encoded enzyme, penicillinase, which hydrolysed penicillin. *S. aureus* has shown a strong adaptive power to antibiotics, first to methicillin, a semi-synthetic derivate of penicillin, through the acquisition of a mobile genetic element (SCC*mec*), and recently to vancomycin through the acquisition of the *van*A gene (53, 54).

STAPHYLOCOCCAL CASSETTE CHROMOSOME *MEC*

The resistance of *S. aureus* to methicillin is caused by the presence of the *mec*A gene, which encodes the 78-kDa penicillin binding protein (PBP) 2a, which has transpeptidase activity. Normally, β-lactam antibiotics bind to PBPs in the cell wall, resulting in the disruption of the synthesis of the peptidoglycan layer. Consequently, the bacterium dies. Since β-lactam antibiotics cannot bind to PBP2a, synthesis of the peptidoglycan layer is not disrupted and cell wall synthesis continues. Consequently, the bacterium survives and becomes resistant to β-lactam antibiotics. The *mec*A gene is regulated by both the repressor MecI and the transmembrane β-lactam-sensing signal-transducer MecR1, which are both divergently transcribed. In the absence of a β-lactam antibiotic, MecI represses the transcription of both *mec*A and *mec*R1-*mec*I, but in the presence of a β-lactam antibiotic, MecR1 is auto-catalytically cleaved and a metalloprotease domain, that is located in the cytoplasmic part of MecR1, becomes active. The metalloprotease cleaves MecI bound to the operator region of *mec*A, which allows transcription of *mec*A and production of PBP2a to occur (5). Both *mec*I and *mec*R1 can be truncated by the insertion sequences IS*431* or IS*1272* and this result in a de-repression of the *mec*A gene (44).

The 2.1-kb *mec*A gene is located on a mobile genetic element, which is designated the Staphylococcal Cassette Chromosome *mec* (SCC*mec*) (39). Currently, five main types of SCC*mec* (type I to V) are distinguished, ranging in size from 20.9 to 66.9 kb (Figure 1). SCC*mec* types I, II and III (34.3, 53.0 and 66.9 kb, respectively) are associated with hospital-acquired MRSA (HA-MRSA), whereas types IV and V (20.9 to 24.3 and 28 kb, respectively) are associated with community-acquired MRSA (CA-MRSA) (16, 36, 37, 38). SCC*mec* types I, IV or V exclusively encode for resistance to β-lactam antibiotics. By contrast, SCC*mec* types II and III determine multi-resistance as these cassettes carry both integrated plasmids (e.g. pUB110, pI258 and/or pT181) and transposon Tn*554* containing drug resistance genes. Integrated plasmid pUB110 carries the *ant*(4') gene, responsible for resistance to aminoglycosides. pI258 codes for resistance to penicillins and heavy metals. Plasmid pT181 codes for tetracycline resistance, while transposon Tn*554* carries the *erm*A gene, which is responsible for inducible MLS resistance (Figure 1) (39, 48). SCC*mec* also carries insertion sequences, such as IS*431* and IS*1272*, and they have a key role in the insertion of the above mentioned plasmids and transposon. Also situated on SCC*mec* are the genes responsible for the regulation of the transcription of *mec*A: Δ*mec*R1 (on SCC*mec* types I, IV and V), *mec*R1 and *mec*I (on SCC*mec* types II and III) (16, 37, 38, 39). These genes are situated in *mec* complexes and four *mec* complexes have been identified:

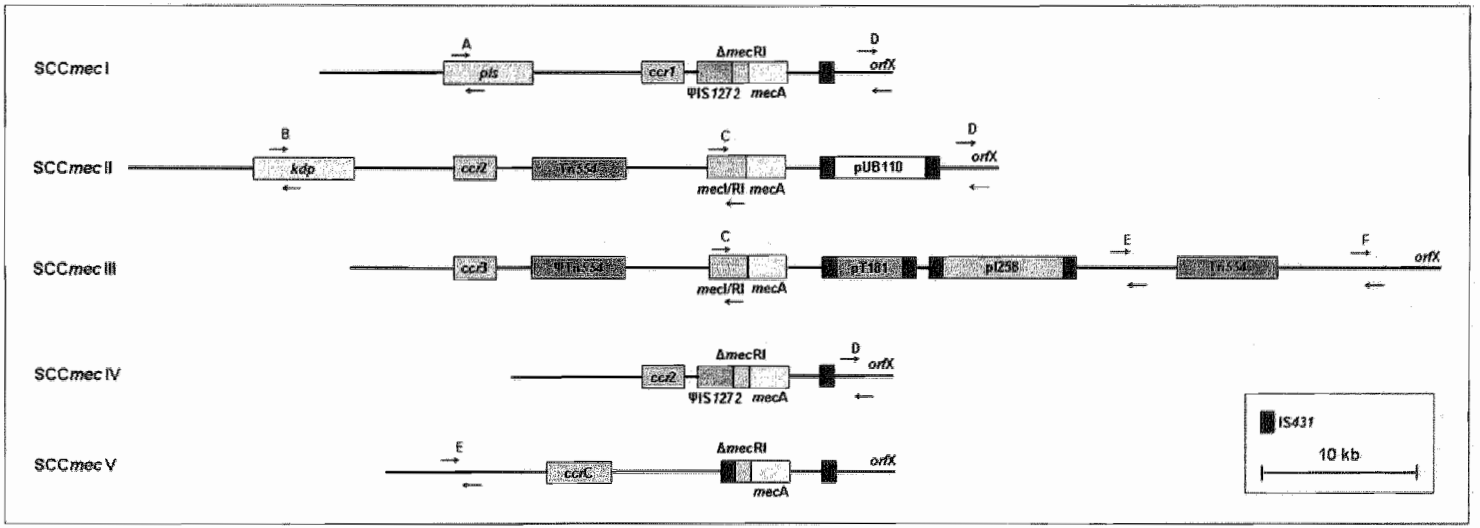

Figure 1. Schematic drawing of SCC*mec* type I to V. The major elements (*ccr* genes, IS*431*, IS*1272*, *mec*A, *mec*I/R1, *orf*X, pI258, pT181, pUB110 and Tn*554*) of the five SCC*mec* types are given as are the six different loci (A to F) used for the typing of SCC*mec* according to the method of Oliveira and de Lencastre (67). The primers of the PCR for the six different SCC*mec* loci are indicated by arrows.

class A: *mecI-mecR1-mecA-IS431* (in SCC*mec* types II and III); class B: IS*1272*-Δ*mec*R1-*mecA*-IS*431* (in SCC*mec* types I and IV), class C: IS*431*-Δ*mec*R1-*mecA*-IS*431* (in SCC*mec* type V) and class D: Δ*mec*R1-*mecA*-IS*431* (in methicillin-resistant *S. caprae*) (37, 38, 44).

For integration into and excision from the chromosome at a specific site (*attBscc*), genes encoding cassette chromosome recombinases (*ccr*) are located within the SCC*mec* elements. These genes are designated *ccr*A1 and *ccr*B1 (in SCC*mec* type I), *ccr*A2 and *ccr*B2 (in SCC*mec* types II and IV), *ccr*A3 and *ccr*B3 (in SCC*mec* type III), *ccr*A4 and *ccr*B4 (in SCC*mec* type IV of MRSA strain HDE288) and *ccr*C (in SCC*mec* type V) (16, 36, 38, 44, 68). The regions bordering the *mec* and *ccr* complexes, integrated plasmids and transposons are designated the J (junkyard) regions (Figure 1) (38).

SCC*mec* was probably transferred from other staphylococcal species to *S. aureus*, but the origin of SCC*mec* is still unknown. *S. epidermidis* could be a source of SCC*mec*, since it has been shown that it harbours SCC*mec* type I to IV (104). It has also been demonstrated that an epidemic methicillin-susceptible *S. aureus* (MSSA) and, subsequently, an isogenic MRSA strain were isolated from a neonate who had never been in contact with MRSA. The *mec*A gene was identical to that of a *S. epidermidis* strain isolated from the same patient. It was concluded that the isolated MRSA strain had emerged *in vivo* by horizontal transfer of *mec*A between staphylococcal species (103). In a further study, a PBP homologue with 87.8% amino acid homology with PBP2a was identified in *S. sciuri* strains and this could be a precursor of PBP2a in MRSA. These strains were all susceptible to methicillin, but upon growth of the strains in the presence of methicillin, they became resistant to methicillin through an increase in the rate of transcription of the *mec*A homologue, due to a point mutation in the promoter. Furthermore, upon introduction of this *mec*A homologue into MSSA, it became resistant to methicillin and could thus be classified as MRSA (106).

The first MRSA strain (NCTC10442) isolated in 1961 in the United Kingdom (UK) harboured SCC*mec* type I and this Archaic clone spread around the world in the 1960s. In 1982, an MRSA strain (N315) with SCC*mec* type II was discovered in Japan and this New York/Japan clone spread worldwide, followed in 1985 by the discovery of an MRSA strain (82/2082) harbouring SCC*mec* type III in New Zealand. MRSA strains harbouring SCC*mec* IV spread around the world in the 1990s and at the beginning of the 21st century the first MRSA strain (WIS) with SCC*mec* type V was isolated in Australia (38, 97, 98).

THE EVOLUTION OF HOSPITAL-ACQUIRED MRSA

Methicillin was first introduced in 1959, but two years later, in 1961, MRSA strains, harbouring SCC*mec* type I, were isolated in the UK. Since then, MRSA has disseminated to other European countries in the 1960s, and thereafter, in the 1970s, to Australia, Japan and the USA. Currently MRSA is a major cause of nosocomial infections worldwide. The worldwide spread of MRSA is driven by the dissemination of a number of clones with a specific genetic background, adapted to virulence and the hospital environment (Table 1) (11, 17, 18, 24, 26, 34, 45, 58, 64, 69, 73, 89, 99). HA-MRSA is characterised by SCC*mec* type I, II or III and most of the HA-MRSA strains have a multi-resistant pattern, although a recent report showed that SCC*mec* type IV can also be associated with HA-MRSA (83).

Multilocus sequence typing (MLST) is an excellent tool to investigate the clonal evolution of MRSA. MLST investigates the DNA sequence of highly conserved DNA regions (± 500 bp) from seven *S. aureus* housekeeping genes, i.e. *arc*C, *aro*E, *glp*F, *gmk*, *pta*, *tpi* and *yqi*L. Different sequences are thus assigned as distinct alleles for each housekeeping gene and each isolate is defined by the alleles of the seven housekeeping genes. This results in an allelic profile or Sequence Type (ST). For example, the Iberian clone has the MLST profile 3-3-1-12-4-4-16 and ST247 (http://www.mlst.net). Currently, the MRSA nomenclature is based on the ST and the SCC*mec* type, i.e. ST247-MRSA-I is the Iberian clone harbouring SCC*mec* type I. Furthermore, clonal complexes (CCs) can be defined with the software package BURST (Based Upon Related Sequence Types) to analyze evolutionary events (http://www.mlst.net). *S. aureus* strains are grouped within one CC when 5 of the 7 housekeeping genes have the same sequence and the ancestor of each CC is the ST with the largest number of single locus variants (SLVs). Sub-group founders can be described as SLVs or double locus variants (DLVs) of a founder of a CC that has become prevalent in a population and may be diversified to produce its own set of SLV and DLVs (23, 24, 25, 90).

Two opposing theories have been suggested to describe the relationship between the first MRSA isolated and recent MRSA clones. The single-clone theory suggested that all MRSA clones have a common ancestor and that SCC*mec* was introduced only once in *S. aureus* (46). The multi-clone theory suggested that SCC*mec* was introduced several times into different genetic *S aureus* lineages and this hypothesis is supported by studies of Musser *et al* using multilocus enzyme electrophoresis (MLEE), Fitzgerald *et al* using DNA micro-array analyses, and Enright *et al* using MLST (24, 27, 60). In the latter study, it was reported that five clonal

Table 1. Overview of the major MRSA clones

Clone	MLST profile	ST[a]	CC[b]	SCC*mec*	Geographic spread[c]
Archaic	3-3-1-1-4-4-16	250	8	I	Den, Ger, Swi, Uga, UK
Southern Germany	1-4-1-4-12-24-29	228	5	I	Bel, Den, Ger, Slo
UK EMRSA-3	1-4-1-4-12-1-10	5	5	I	Nor, Pol, Slo, UK
Iberian	3-3-1-12-4-4-16	247	8	I	Bel, Cze, Den, Fin, Fra, Ger, Ita, Net, Pol, Por, Slo, Spa, Swe, Swi, UK, USA
Irish-1	3-3-1-1-4-4-3	8	6	II	Ire, UK, USA
New York/Japan	1-4-1-4-12-1-10	5	5	II	Bel, Can, Den, Fin, Ire, Jap, Kor, Mex, UK, USA
UK EMRSA-16	2-2-2-2-3-3-2	36	36	II	Bel, Can, Den, Fin, Gre, Mex, Nor, Spa, Swe, Swi, UK, USA
Brazilian/Hungarian	2-3-1-1-4-4-3	239	8	III	Arg, Aus, Bra, Chi, Chn, Cze, Fin, Ger, Gre, Ind, Ids, Net, Pol, Por, Sin, Slo, Sri, Swe, Tha, UK, Uru, USA, Vie
Berlin	10-14-8-6-10-3-2	45	45	IV	Bel, Fin, Ger, Net, Nor, Swe, USA
Pediatric	1-4-1-4-12-1-10	5	5	IV	Arg, Ast, Col, Den, Fra, Kor, Nor, Pol, Por, Spa, UK, USA
UK EMRSA-2/-6	3-3-1-1-4-4-3	8	8	IV	Ast, Bel, Fin, Fra, Ger, Ire, Net, Nor, Tai, UK, USA
UK EMRSA-15	7-6-1-5-8-8-6	22	22	IV	Ast, Bel, Cze, Ger, Den, Ire, Nor, Spa, Swe, UK

[a]Sequence Type, [b]Clonal Complex, [c]Arg-Argentina, Ast-Australia, Aus-Austria, Bel-Belgium, Bra-Brazil, Can-Canada, Chi-Chile, Chn-China, Col-Colombia, Cze-Czech Republic, Den-Denmark, Fin-Finland, Fra-France, Ger-Germany, Gre-Greece, Ind-India, Ids-Indonesia, Ire-Ireland, Ita-Italy, Jap-Japan, Kor-Korea, Mex-Mexico, Net-Netherlands, Nor-Norway, Pol-Poland, Pro-Portugal, Sin-Singapore, Slo-Slovenia, Sri-Sri Lanka, Spa-Spain, Swe-Sweden, Swi-Switzerland, Tai-Taiwan, Tha-Thailand, Uga-Uganda, UK-United Kingdom, Uru-Uruguay, USA-United States of America, Vie-Vietnam

complexes were found among 359 MRSA strains isolated in 20 countries between 1961 and 1999 and that strains with the same ST harboured different SCC*mec* types (24).

To investigate the relationship between MSSA and MRSA, a study was performed by Crisostomo *et al.* They reported that ST250 was found predominantly among MSSA strains isolated in Denmark in the 1950s. It was concluded that ST250 could be the ancestor of the first MRSA strain isolated in the UK in 1961 (14). Enright *et al* further investigated MSSA and MRSA strains from the UK. A high level of genetic similarity was found between MSSA (ST30) and MRSA (ST36). The difference between ST30 and ST36 is a point mutation in the *pta* gene. It was concluded that ST36-MRSA-II (EMRSA-16) has emerged from ST30-MSSA through ST36-MSSA upon acquisition of SCC*mec* type II (23).

To further investigate the evolution of MRSA, SCC*mec* typing, together with MLST and BURST analyses was applied on 359 MRSA and 553 MSSA strains isolated between 1961 and 1999 from 20 countries worldwide. It was shown that the major MRSA clones, defined as groups of isolates from more than one country with the same ST and SCC*mec* type, belonged to one of five clonal complexes (CC5, 8, 22, 30, and 45). As shown in table 1, different SCC*mec* types have been acquired by *S. aureus* strains with the same genetic background, i.e. ST5-MSSA has acquired SCC*mec* types I to IV, to form ST5-MRSA-I to IV. Furthermore, it was shown that ST8-MSSA in CC8 is the ancestor of the first MRSA, as ST250-MRSA-I was derived from ST250-MSSA, which arose from ST8-MSSA through a point mutation in the *yqi*L gene. ST8-MSSA is a common cause of epidemic MSSA disease and it has acquired SCC*mec* types I and IV. Derived from ST250 by a point mutation at the *gmk* locus is ST247-MRSA-I, the Iberian clone. This is one of the major MRSA clones currently isolated in European hospitals. Another major MRSA ST within CC8 was ST239 (ST239-MRSA-III), which corresponds to the Brazilian clone. It is derived from ST8-MRSA-III by recombination in the *arc*C gene. It was further shown that CC5, 22, 36 and 45 were all derived from epidemic lineages that have acquired SCC*mec*, since they differed from each other and from ST8 at six or seven loci (table 1). Furthermore, these MLST analyses showed that some of the first vancomycin-intermediate *S. aureus* (VISA) isolates have emerged from ST5-MRSA-II, a pandemic MRSA clone known as the New York/Japan clone (24). A recent study confirmed that multiple lineages of *S. aureus* harbour different SCC*mec* types and that both CA-MRSA and HA-MRSA strains were found among hospitalized patients in Australia (12).

Another study of 147 geographically diverse MRSA strains showed that MRSA has emerged at least twenty times upon acquisition of SCC*mec* and that the acquisition

of SCC*mec* by MSSA was four times more common than the replacement of one SCC*mec* with another type. Interestingly, SCC*mec* type IV was found in twice as many MRSA clones than other SCC*mec* types. This suggested that most clones arise by acquisition of SCC*mec* type IV in *S. aureus* (79).

Recently, two studies were published in which clonal evolution within one hospital was described. ST30-MRSA-IV was present in a Mexican hospital between 1997 and 2000, but this clone was replaced by ST5-MRSA-II in 2001 and was dominating in 2002 (99). Another study in Spain showed that between 1998 and 2002, ST247-MRSA-I was replaced by ST36-MRSA-II (73). Besides the major clones shown in table 1, MRSA strains are also isolated in single hospitals (minor clones) or from single patients (sporadic isolates) (17). Although most of the MRSA strains are isolated in hospitals, community-acquired strains have recently emerged.

THE EMERGE OF COMMUNITY-ACQUIRED MRSA

The first CA-MRSA strain was reported in Western Australia in 1993 from hospitalised patients who lived in remote communities (96). During the 1990s, CA-MRSA strains spread worldwide (98). Interestingly, CA-MRSA strains are isolated from patients who have no or few risk factors associated with MRSA colonisation. It was found that CA-MRSA is both genotypically and phenotypically different from HA-MRSA. In contrast to HA-MRSA, CA-MRSA strains are largely susceptible to antibiotics other than β-lactam antibiotics. Pulsed-Field Gel Electrophoresis (PFGE) analyses have shown that CA-MRSA belongs to a single clonal type and that this clone is unrelated to clones isolated in hospitals (33, 62). Moreover, SCC*mec* types IV and V are associated with CA-MRSA (16, 38, 39). Furthermore, many CA-MRSA strains harbour PVL and several clones, as shown by MLST, are disseminated worldwide (98). However, MRSA strains harbouring SCC*mec* type IV have also been associated with nosocomial infections (65, 83). This could suggest that MRSA strains harbouring SCC*mec* type IV are adapted to both the hospital and the community environment. This is probably due to the smaller size of SCC*mec* type IV, which facilitates easier transfer between staphylococcal species, compared to other SCC*mec* types (80). One report described two CA-MRSA strains harbouring SCC*mec* type II (11).

Okuma *et al* investigated the origin of CA-MRSA, e.g. whether CA-MRSA strains are HA-MRSA with SCC*mec* type IV, or MSSA that have acquired SCC*mec* type IV in the community. For this study, a total of 23 CA-MRSA isolates from Australia and the USA was investigated by MLST. They found that the two main STs were ST1 and ST30. *S. aureus* with ST1 is normally found in the community, but it was not

associated with CA-MRSA until this study was performed. ST30 is usually only found in MSSA, but only a few HA-MRSA with this ST were found, but they did not harbour SCC*mec* type IV. This study showed that CA-MRSA strains are truly novel acquisitions of SCC*mec* type IV in the community (66). Furthermore, it was shown that CA-MRSA has greater clonal diversity than HA-MRSA, since seven CCs (CC1, 5, 8, 22, 30, 45, and 298) were found among the 23 strains investigated, compared to five CCs among 359 HA-MRSA strains (24, 66). The emerge of SCC*mec* type IV in virulent strains with a ST1 and ST30 background is very worrying, since these strains also harbour the genes coding for PVL (31, 98).

Later on, another study by Aires de Sousa *et al* (1) raised the possibility that some CA-MRSA strains may actually originate in hospitals. This study showed interesting similarities between CA-MRSA and HA-MRSA strains. A total of 41 HA-MRSA strains that differed from the major pandemic clones by PFGE pattern, *mec*A and Tn*554* polymorphism, as well as epidemic behaviour were selected from an international strain collection. MLST, *spa* typing, and SCC*mec* typing showed extensive diversity among these sporadic isolates, both in the genetic background and the SCC*mec* type. Nevertheless, the isolates could be grouped into restricted CCs (CC5, 8, 22, 30, 45, and 91) by BURST, which showed that most sporadic MRSA isolates evolved from pandemic MRSA clones. Several of these HA-MRSA strains resembled CA-MRSA isolates in properties that include a limited multi-resistance pattern, faster growth rate, diversity of the genetic background, and carriage of SCC*mec* type IV (1).

CA-MRSA strains from Europe differ from Australian and US strains in their PFGE pattern, MLST profile (ST1 in US isolates, ST30 in Australian isolates, and ST80 in European isolates), and SCC*mec* type (98). European CA-MRSA harbour *ccr*AB2, the class B *mec* complex, but they could not be typed with SCC*mec* type IV specific primers. Furthermore, they harboured the *far*I gene, encoding for resistance to fusidic acid and have a unique *spa* type (r07-r23-r12-r34-r34-r33-r34). This clone is spread through Finland, France, Germany, Norway, and Scotland (22, 105). All three CA-MRSA clones carry the genes for the production of PVL, *luk*F-PV and *luk*S-PV. SCC*mec* type V was present in Australian CA-MRSA strains with ST5, 45 and 152 (12).

A worrying trend is the transmission of MRSA from humans to pets as was described recently. An MRSA strain with a novel SCC*mec* type was isolated from a dog and the owner, who worked in a nursing home. This study showed that the spread of MRSA from health care facilities to the community could occur trough pets (97).

VIRULENCE FACTORS

The ability of *S. aureus* to cause infections is mainly attributed to the production of virulence factors, such as Panton-Valentine leukocidin (PVL) and the family of the superantigens (SAgs). The genes for the production of these virulence factors are mainly situated on genomic islands (GIS) and both MSSA and MRSA can harbour these virulence genes. It is believed that the genes for the production of SAgs were acquired from *Streptococcus pyogenes*, another human pathogen producing different SAgs (74).

Panton Valentine leukocidin

Panton-Valentine leukocidin (PVL) is a *S. aureus*-specific exotoxin that belongs to the family of bicomponent synergohymenotropic toxins (32, 92). It is encoded by two contiguous and cotranscribed genes, designated *luk*F-PV and *luk*S-PV (77), which produce proteins of 32 and 38 kDa respectively. Like all other leukocidins, PVL kills leukocytes by creating pores in the cell membrane. Purified PVL was reported to induce severe inflammatory lesions when injected intradermally in rabbits, leads into capillary dilation, infiltration of polymorphonuclear cells, and skin necrosis (77, 101).

The genes encoding PVL, which are carried on a bacteriophage, are found in only a subset of *S. aureus* isolates. Prévost *et al* reported that PVL was harboured by fewer than 5% of the isolates in a general hospital (77). In accordance with the aforementioned activities of purified PVL, PVL-positivity of *S. aureus* strains was found to be associated with primary skin infections such as furunculosis, and severe necrotizing pneumonia (31, 49). The clinical importance of PVL was further demonstrated in a study of *S. aureus* infections which occurred between 1986 and 1998 in France. This study revealed eight cases of community-acquired pneumonia caused by PVL-positive *S. aureus* strains, six of which with a fatal outcome. The patients involved were all immunocompetent children and young adults, and each of them presented with a preceding influenza-like syndrome before developing pneumonia (31).

Furthermore, Vandenesch *et al* performed a study in which the presence of 24 virulence factors and SCC*mec* in 117 CA-MRSA isolates from a large number of countries worldwide was investigated. This investigation showed that CA-MRSA was characterized by SCC*mec* type IV and that PVL was a stable genetic marker for CA-MRSA (98). The relationship between CA-MRSA, SCC*mec* type IV and PVL (ST1-MRSA-IV) was confirmed by a study by Shukla *et al* in the USA (89). However, another

study by O'Brien *et al* in Australia did not find a relationship between CA-MRSA, SCC*mec* type IV and PVL (64).

Superantigens

The pathogenicity of *S. aureus* can in part be attributed to the production of pyrogenic toxins. Because secretion of these toxins causes excessive stimulation of T lymphocytes, they are called superantigens (SAgs) (57). To date, several different SAgs have been identified in *S. aureus*, i.e. staphylococcal enterotoxins (SE) A to U, exfoliative toxins (ET) A and B, and toxic shock syndrome toxin-1 (TSST-1) (2, 43, 74). However, only a small number of SAgs is produced by each *S. aureus* strain. Several studies have shown that SAgs play a role in food poisoning, toxic shock syndrome (TSS), scarlet and rheumatic fever, arthritis, multiple sclerosis, diabetes, sudden infant death syndrome (SIDS) and Kawasaki syndrome (29, 52).

The potency of SAgs lies in their ability to efficiently induce T-cell proliferation and activation (10,000-fold more efficiently than other antigens) by bridging the antigen-presenting cells (APC) and T-cell receptors (TCRs) through binding the major histocompatibility complex (MHC) class II on APCs and specific variable regions on the β-chain (TCR Vß) of both CD4 and CD8 antigen receptors. Consequently, a T-helper 1 type response is mounted, which results in the massive release of interleukins and pro-inflammatory cytokines, such as tumour necrosis factor-α (TNF-α), interleukin-2 (IL-2) from the APCs and interferon-γ (INF-γ) from the T-cells (20, 21, 28, 47, 72). Furthermore, SAgs are capable of inducing cytotoxicity towards target cells (74).

The genes responsible for the production of SAgs are situated on plasmids, on prophages, or on *S. aureus* pathogenicity islands (SaPI). Currently, seven SaPIs have been identified. These seven SaPIs can be classified into four groups, depending on their integrase homology and insertion site. In addition, it has been found that no more than one SaPI is present in an *S. aureus* strain. The SaPIs are packed into phage heads, and are integrated into the chromosome of *S. aureus*. This implies that they can move with a high frequency between *S. aureus* strains (6, 50, 51).

The SAgs that have been structurally characterized share three-dimensional folds, despite their low amino acid sequence similarity. It is assumed that SAgs have a common ancestor and that horizontal transfer, together with vertical evolution are responsible for the diversity among SAgs. The structure of SAgs shows conserved two-domain architecture (N- and C-terminal domains) and a long solvent-accessible α-helix spanning the centre of the molecule. The N-terminal domain is characterized by the presence of hydrophobic residues in solvent-exposed regions and the C-terminal

domain comprises a four-stranded ß-sheet capped by a long α-helix (70, 85, 91, 93). Zinc ions are important for the three-dimensional stability of most SAgs, as well as their function, and their ability to bind MHC class II (74). Both SEB and TSST-1 do not bind zinc (76, 93).

Staphylococcal enterotoxins A, B, C1 to C3, D, E, G to R and U are 24.8 to 31.2 kDa polypeptides and are mainly responsible for food poisoning. SEH however, plays a role in TSS. These enterotoxins belong to a family of heat stable enterotoxins and show 50 to 85% homology in their predicted amino acid level. Furthermore, SEA is a potent gastrointestinal toxin, while Inhalation of SEB can induce pathophysiological changes, which include widespread systemic damage and septic shock (29, 30, 52, 74, 78).

The exfoliative toxins (ET) A (26.9 kDa) and B (27.3 kDa) are responsible for staphylococcal scalded-skin syndrome (SSSS), an exfoliative dermatitis of infants and children, that is characterized by the formation of large bullae without inflammatory cell infiltrate and the separation of extended areas of the epidermis at the stratum granulosum leaving the keratinocytes intact. ETA is chromosomal encoded, whereas ETB is plasmid encoded. These proteins share a 40% homology (40, 41, 107).

TSST-1 is a 29.1 kDa protein that is encoded by the *tst* gene. This gene is present in up to 20% of the *S. aureus* strains (29, 52). The release of TSST-1 into the bloodstream may give rise to a variety of severe clinical conditions, such as TSS, SIDS, and Kawasaki syndrome. TSS is characterized by high fever, erythematous rash formation, hypotension and major oxygen involvement, which may lead to multi organ failure. Without the appropriate therapy, a lethal shock may develop within 24 hours after the onset of symptoms (20, 54, 57). Although most cases (two thirds) of TSS are associated with tampon use, an increasing number of cases are related to localized infections, surgical complications and insect bites (54). Musser *et al* showed that the majority of female patients affected with TSS had a single *S. aureus* clone well adapted to colonization in the genital tract (61). TSS is usually treated with proper drainage of surgical wounds, a high dose of a β-lactam antibiotic and an γ-globulin (3). Nevertheless, TSS still has a lethality rate of about 30% (29).

PREVALENCE AND RISK FACTORS

A high prevalence of MRSA in hospitals has been associated with higher patient mortality and higher health care costs. The SENTRY study indicated that the prevalence of MRSA in hospitals worldwide between 1997 and 1999 was 22.4% in Australia, 66.8% in Japan, 34.9% in Latin America, 40.4% in South America, 32.4% in

the USA, and 26.3% in Europe (4, 19). The prevalence of MRSA between countries in Europe varies. The prevalence of MRSA in the northern countries (0.6% in the Scandinavian countries and The Netherlands) is lower than in most other European countries (up to 44.7%). This low prevalence of MRSA in North Europe is attributed to a low antibiotic pressure and screening of 'high-risk' patients for MRSA before they enter a hospital (Search-and-Destroy) (95). Risk factors for MRSA colonisation include previous exposure to one or more antibiotics, prolonged duration of therapy, stay in an intensive care unit or a burn unit, severe underlying illness, invasive procedures, surgical wounds or burns and contact with patients colonised with MRSA (7, 8, 15, 42, 71, 102). It has also been shown that if a patient, who receives antibiotic treatment, acquired MRSA, it survived and proliferated more easily than MSSA, because it had a selective advantage (86).

Furthermore, more effective disinfection procedures and hand hygiene guidelines would partly help to prevent spread of MRSA in a hospital environment (75). Strict implementation of the above rules in Denmark 25 years ago, when the prevalence of MRSA was as high as 30%, have decreased the MRSA prevalence to less than 1% and this low percentage has been maintained since then (81).

A least eight definitions are known for CA-MRSA (63, 84), but the Center for Disease Control and Prevention (CDC) defines CA-MRSA as strains isolated within 48 hours of admission to a hospital. The prevalence of CA-MRSA is still low worldwide, but a recent report indicates that the prevalence of CA-MRSA is increasing (84). Several studies have been performed and they showed that the prevalence of CA-MRSA is lower than 0.5%. In a study among 500 children and their guardians in New York, one child was colonised with MRSA. This corresponded with a CA-MRSA prevalence of 0.2%. This child, which had been hospitalised several times within the last year, was found to be colonised with an MRSA strain identified as the predominant clone found in hospitals in New York (87). A further study in the San Francisco area among 833 homeless and marginally housed adults showed that 23 adults were colonized with MRSA (2.8%), but further investigation showed that only 2 of the 23 adults had no risk factors for MRSA colonization, so that the prevalence of CA-MRSA was as low as 0.24% (9). A study performed in Portugal showed that among 3,525 healthy and young individuals, without risk factors for MRSA colonization, only 7 were colonized with CA-MRSA (0.2%). Of the seven CA-MRSA strains, five were identified as either the Brazilian, Iberian, or Pediatric clone, which are endemic in Portuguese hospitals (82). Furthermore, Salgado *et al* performed a global analysis of 57 studies on CA-MRSA prevalence among both hospitalised patients and community members, and reported that most persons with CA-MRSA had at least one risk factor for MRSA. This

study suggested that the prevalence of CA-MRSA among persons without risk factors is 0.24% (84). A recent study showed that the prevalence of CA-MRSA in Europe is 0.03 to 1.5% (95). The high rates of MRSA colonisation among Australian aboriginals or Native Americans is probably associated with risk factors for spread in the community, such as skin infections and use of broad-spectrum antibiotics (33, 56). In other studies, a number of risk factors associated with CA-MRSA colonisation were identified. These included gastrointestinal disease, recent transmission to a hospital and injection drug use. Furthermore, it was shown that recent medication tends also to be a risk factor for CA-MRSA colonisation (10, 55).

Any strategy to contain the spread of MRSA, either in the community, in a hospital, at national or at international level, requires a throughout knowledge how MRSA is disseminate, the number of clones that are spread and the relatedness of these clones to each other. Today, the epidemiology of MRSA is investigated with PFGE, SCC*mec* typing, and MLST (17). First, the PFGE patterns are analyzed with Dice comparison and unweighted pair group matching analysis (UPGMA) settings according to the scheme of Tenover *et al* (94). The position tolerance is set at 1 to 2% and isolates with a similarity index of 0.80 or more are classified as a clonal group. A small selection of strains from the major clonal groups is then further investigated by MLST and SCC*mec* typing (12, 73).

FUTURE PROSPECTS

Rapid and accurate molecular characterisation of MRSA, based on the SCC*mec* type and the investigation of the genetic background by MLST, is still a labour intensive task. Two methods are described in the literature to investigate the type of SCC*mec*. The first method of Oliveira *et al* is a multiplex PCR method, where *mec*A and six different loci on SCC*mec* are amplified with PCR (67). The second method by Ito *et al* characterise the SCC*mec* type based on the combination of the *mec* complex and the *ccr* genes (38, 39). The first method is used in most studies, and the method of Ito *et al* is now used when the SCC*mec* type cannot be determined with the method of Oliveira *et al*. However, the two methods gave different results when typing SCC*mec* (88). A general and international agreement should be reached to define known and future SCC*mec* types, and than one method should be developed and used in future studies. This method should be evaluated on all major MRSA clones as investigated by MLST (24). Since MLST is very labour intensive, a novel molecular typing method for the investigation of the dissemination of MRSA should be developed, that brings with it the speed of *spa* typing and the accuracy of MLST. This method should ideally be a

combination of MLST, SCC*mec* typing and should also include the determination of *S. aureus* specific genes, such as *fem*A, and several important virulence factors, like PVL.

The worldwide emerge of CA-MRSA is a threat to both the community and the hospital environment, since these strains are known to be more virulent than HA-MRSA strains. First, a clear definition for CA-MRSA, instead of at least eight definitions that are now available (63, 84), must be implemented in research. Second, studies to investigate the prevalence of CA-MRSA and their genetic background should be started. MLST analyses are necessary to investigate if these strains have for example a ST80 background, the common CA-MRSA background found in Europe. Furthermore, in these studies, the presence of a larger panel of virulence factors should be investigated by real-time PCR. This study should also focus on determining the risk factors for CA-MRSA infection. Another intriguing question concerning CA-MRSA is the possible relation between SCC*mec* type IV (and V) and PVL, since reports are conflicting as to whether there is a relation between SCC*mec* type IV and PVL (11, 64, 98). This should be performed with a real-time PCR assay, combining the *S. aureus* specific *fem*A gene, the resistance gene *mec*A, *ccr*AB2, Δ*mec*R1 (in combination specific for SCC*mec* type IV) and PVL. CA-MRSA strains with a different genetic background as investigated by MLST should be studied (24, 98).

There are still a number of intriguing questions unanswered concerning the molecular evolution of MRSA. One of the most intriguing is the origin of SCC*mec*. In this respect, it is noteworthy that Hanssen *et al* studied 39 methicillin-resistant coagulase-negative staphylococci (MRCNS), and 22 had a novel SCC*mec* type (35). These novel SCC*mec* types could give us more information about the possible transfer of SCC*mec* between CNS species and *S. aureus*. Moreover, other studies found novel SCC*mec* types (88, 97, 100), or SCC elements without *mec*A, which could be a reservoir for antibiotic resistance islands, in *S. aureus* (13, 59). Further studies should also investigate the role of pets and farm animals in the transfer of SCC*mec*, i.e. is SCC*mec* formed in animals in MRCNS or MRSA and transferred to humans, or are humans the source of CNS or *S. aureus* strains carrying SCC*mec*?

It has been suggested that MRSA is more pathogenic than MSSA, but little evidence has been brought forward, except that CA-MRSA, harbouring SCC*mec* type IV and PVL, have caused necrotising pneumonia in young adults. However, a relation between resistance and virulence cannot be ruled out, since it has been shown that sub-inhibitory concentrations of antibiotics influence the expression of virulence factors.

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CHAPTER 3

Rapid detection of Panton-Valentine leukocidin from clinical isolates of *Staphylococcus aureus* strains by real-time PCR

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ABSTRACT

To allow rapid identification of Panton-Valentine leukocidin (PVL)-producing *Staphylococcus aureus* strains, a real-time PCR assay for detection of PVL was developed. This assay is convenient, since it can be applied directly on bacterial suspensions and does not require previous DNA purification. Furthermore, the assay was found to be highly reproducible, robust and specific, since positive results were generated exclusively with PVL-positive *S. aureus* strains, and neither with PVL-negative strains nor staphylococci other than *S. aureus*.

INTRODUCTION

Staphylococcus aureus is a potential pathogenic micro-organism that can cause various diseases, like pneumonia, toxic shock syndrome (TSS) and wound infections (1). Panton-Valentine leukocidin (PVL) is a *S. aureus*-specific exotoxin that belongs to the family of bicomponent synergohymenotropic toxins (4, 10). PVL can be harboured in both methicillin-susceptible *S. aureus* (MSSA) and methicillin-resistant *S. aureus* (MRSA) strains. It is encoded by two contiguous and cotranscribed genes, designated *luk*F-PV and *luk*S-PV (8). Like all other leukocidins, PVL kills leukocytes by creating pores in the cell membrane. Purified PVL was reported to induce severe inflammatory lesions when injected intradermally in rabbits, leading to capillary dilation, infiltration of polymorphonuclear cells, and skin necrosis (8, 13).

The genes encoding PVL, which are carried on a bacteriophage, are found in only a subset of *S. aureus* isolates. Prévost *et al.* reported that PVL was harboured by fewer than 5% of the isolates in a general hospital (8). In accordance with the aforementioned activities of purified PVL, PVL-positivity of *S. aureus* strains was found to be associated with primary skin infections, such as furunculosis, and severe necrotizing pneumonia (3, 6). The clinical importance of PVL was further demonstrated in a study of *S. aureus* infections which occurred between 1986 and 1998 in France. This study revealed eight cases of community-acquired pneumonia caused by PVL-positive *S. aureus* strains, six of which with a fatal outcome. The patients involved were all immunocompetent children and young adults, and each of them presented with a preceding influenza-like syndrome before developing pneumonia. (3).

Furthermore, during the last years infections caused by community acquired MRSA (CA-MRSA) have been an emerging phenomenon worldwide. Vandenesch *et al* performed a study in which the presence of 24 virulence factors and the methicillin resistance determinant (SCC*mec*) in 117 CA-MRSA isolates from a large number of

countries worldwide was investigated. This investigation showed that CA-MRSA was characterized by SCC*mec* type IV and that PVL is a stable genetic marker for CA-MRSA (11).

To investigate the prevalence of PVL-producing *S. aureus* isolates in our hospital and the community, and to allow monitoring of patients for PVL-producing strains, we developed a real-time PCR (TaqMan®) assay for the rapid detection of PVL. Rapid detection of PVL in clinical isolates of MSSA and MRSA may have consequences for the antibiotic therapy prescribed.

MATERIALS AND METHODS

Bacterial isolates

One-hundred characterized *S. aureus* strains consisting of 51 PVL-negative (30 PVL-negative MSSA and 21 PVL-negative MRSA) and 49 PVL-positive (23 PVL-positive MSSA and 26 PVL-positive MRSA) *S. aureus* isolates as tested with conventional PCR (6) were used to investigate the specificity of the assay. These strains included different *S. aureus* clones spreading around the world from countries like Australia (MRSA with sequence type (ST) 30 and 93), China (MRSA with ST30), France (MSSA with ST1, 22, 25, 26, 30, 80, 121 and 188 and MRSA with ST8 and 80), the Netherlands (MRSA with ST8, 22, 37, 59 and 377), Spain (MSSA with ST121), Switzerland (MRSA with ST5), Tahiti (MSSA with ST1 and 181) and the United States of America (MRSA with ST1, 8 and 59) (11, 12).

To test the potential cross-reactivity with other staphylococcal species, 20 clinical *Staphylococcus epidermidis* isolates (5 methicillin-susceptible [MSSE] and 15 methicillin-resistant *S. epidermidis* isolates [MRSE]), 3 *S. capitis*, 1 *S. chromogenes*, 1 *S. cohnii*, 4 *S. haemolyticus*, 1 *S. hominis*, 1 *S. sciuri* and 1 *S. warneri* clinical isolates were used.

To investigate the presence of PVL in clinical isolates from our hospital, a total of 106 *S. aureus* isolates from blood cultures, obtained between 1999 and 2003, were tested. We included both community acquired (CA) *S. aureus* isolates (isolated within 48 hours after patient admission to the hospital) and hospital acquired (HA) *S. aureus* isolates (isolated after 72 hours of admission).

MRSA Cluster 28 was used as the reference strain for PVL (12). For the identification of the strains, the catalase and coagulase activity was tested, followed by further identification with the API Staph identification system (Biomérieux, Boxtel, The Netherlands).

Primer and MGB probe design

The primers and the TaqMan®-minor-groove-binding (MGB) probe were designed on the basis of the published sequences of PVL (GenBank accession number AB006796 (5)), using the computer programme Primer Express 2.0 (Applied Biosystems [ABI], Nieuwerkerk a/d Ijssel, The Netherlands). The sequences of the forward and reverse primer were 5'-GCTGGACAAAACTTCTTGGAATAT–3' (PVL-FP, corres-ponding to position 2666-2690 of the PVL sequence (5)) and 5'-GAT AGGACACCAATAAATTCT GGATTG–3' (PVL-RP, corresponding to position 2749-2723 of the PVL sequence (5)), respectively (Sigma-Genosys, Cambridge, United Kingdom). The sequence of the VIC-labelled MGB probe was 5'-AAAATGCCAGTGTTATCCA–3' (PVL-PR, corresponding to position 2694-2712 of the PVL sequence (5)) (ABI, Nieuwerkerk a/d Ijssel, The Netherlands). The specificity of the primer and probe sequences were confirmed by screening of sequence databases using BLAST (http://www.ncbi.nlm.nih.gov/blast).

TaqMan assay for PVL

The assay conditions, such as primer and probe concentrations, were optimized according to the guidelines from both the Primer Express 2.0 software programme and the manual (Protocol) of the TaqMan® Universal PCR Master Mix (ABI, Nieuwerkerk a/d Ijssel, The Netherlands). The following reaction conditions were used: 0.6 µM of PVL-FP, 0.6 µM of PVL-RP, 175 nM of MGB probe, 1 x TaqMan® Universal PCR Master Mix (ABI, Nieuwerkerk a/d Ijssel, The Netherlands) and 20 µl of purified genomic DNA (isolated from a 1 McFarland suspension using the Wizard® Genomic DNA Purification Kit (Promega, Leiden, The Netherlands) (7)) or 20 µl of a 0.5 to 1 McFarland suspension (1.5 to 3 x 10^8 colony forming units [CFU]/ml) of the isolates in a total volume of 50 µl. After amplification (2 min at 50°C, 10 min at 95°C, followed by 42 cycles of 15 sec at 95°C and 60 sec at 60°C) using the ABI PRISM 7000 Sequence Detection System, Ct (threshold cycle) values were calculated.

Results and Discussion

Determination of the detection limit of the PVL real-time PCR assay

Initially, the PVL TaqMan assay was tested using both purified bacterial DNA as well as bacterial suspensions as input material. Since the assay performed similarly

using either of both types of material (data not shown), we optimized the assay using bacterial suspensions directly in the reaction mixtures. To assess the detection limit of the assay, four ten-fold dilutions were made of a 1 McFarland suspension of a PVL-positive *S. aureus* strain. Then, each of the dilutions was tested directly in the PCR assay. As shown in Figure 1a, the four dilutions generated Ct values ranging from 19.5 to 33.5. The standard curve that was derived from these values (Figure 1b) showed a near optimal slope of -3.5, indicating that the assay was optimal over a broad dynamic range. Thus, a signal with suspensions of at least 30 CFU/µl, which corresponds to a 10,000 fold dilution of a 1 McFarland suspension could be detected.

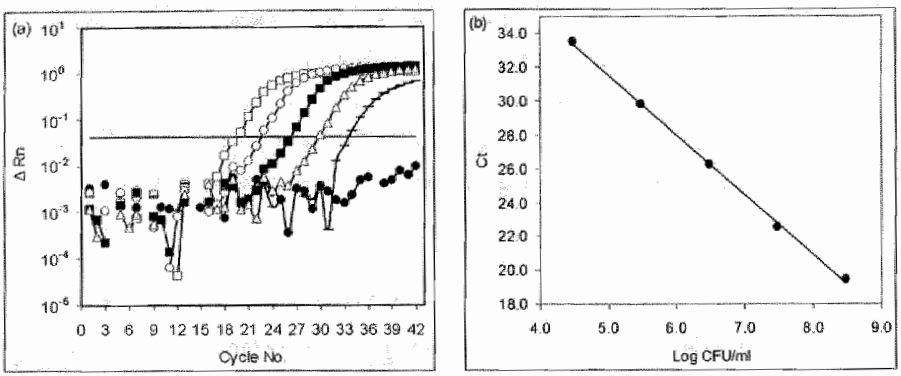

Figure 1. (a) PCR amplification curves of serial dilutions of a PVL-positive *S. aureus* strain. The strengths of the suspensions ranged from 3 x 10^4 to 3 x 10^8 CFU/ml (1 McFarland). Δ Rn indicates the normalized fluorescent reporter value, subtracted from the background value. No Template Count (NTC) (-•-); 3 x 10^8 CFU/ml (-□-); 3 x 10^7 CFU/ml (-o-); 3 x 10^6 CFU/ml (-■-); 3 x 10^5 CFU/ml (-Δ-); 3 x 10^4 CFU/ml (-_-); Threshold (---). (b) Standard curve generated with the Ct values from the amplification curves shown in (a).

Specificity of the assay

To determine the specificity of the assay, a large panel of PVL-positive and PVL-negative *S. aureus* isolates was tested. The PVL-positive strains produced Ct values between 17.46 and 23.76 (mean=20.23, SD=1.72), whereas all PVL-negative strains did not generate detectable signals. Potential cross-reactivity with staphylococcal species other than *S. aureus* was also tested in the assay. None of these 32 isolates generated positive results in the assay (data not shown). These data indicate that the PVL real-time PCR assay was highly specific.

Reproducibility of the assay

The reproducibility of the PCR assay was tested with six PVL-positive *S. aureus* strains in five independent runs. The strains used included MRSA Cluster 28 and five independent MRSA strains from the Netherlands. In the five PCR runs, mean Ct values were found of 19.00 (SD=0.35), 21.43 (SD=0.46), 19.40 (SD=0.32), 18.77 (SD=0.35), 19.02 (SD=0.40) and 20.32 (SD=0.09), respectively. This shows that the real-time PVL PCR was highly reproducible.

Investigation of PVL in clinical isolates

Two of the 63 (3.2%) CA *S. aureus* isolates were positive for PVL and generated Ct values of 22.4 and 19.2, respectively. None of the 43 HA *S. aureus* isolates were positive for PVL. The percentage of PVL-positive strains corresponded to the results found by Prévost *et al.* in a French study, who found a percentage of less than 5% among isolates from a general hospital (8).

Conclusion

In conclusion, a real-time PCR assay was developed for the detection of *S. aureus* strains producing PVL. This assay generated results at least two times faster than the conventional PCR methods previously used for the detection of PVL. The assay was very convenient, since it can be applied directly on bacterial suspensions and does not require previous DNA purification. Furthermore, the assay was found to be highly reproducible, specific and robust. Since PVL-carrying *S. aureus* strains pose an upcoming problem worldwide, both in the hospital and in the community, the availability of a rapid, real-time PVL PCR assay will help to identify PVL-harbouring *S. aureus* strains and thus contribute to the prevention of the spread of such strains. Moreover, infections with PVL positive strains could warrant, like pyrogenic exotoxin producing *Streptococcus pyogenes*, a combination therapy of a penicillin and clindamycin. This combination proved to be more effective than penicillin alone for severe group A streptococci infections (9).

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CHAPTER 4

Different clonal complexes of methicillin-resistant *Staphylococcus aureus* are disseminated in the Euregio Meuse-Rhine

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ABSTRACT

The Euregio Meuse-Rhine (EMR) is formed by the border regions of Belgium, Germany and The Netherlands. Cross-border health care requires infection control measures, in particular since the prevalence of methicillin-resistant *Staphylococcus aureus* (MRSA) differs between the three countries. To investigate the dissemination of MRSA in the EMR, 152 MRSA isolates were characterized by Pulsed-Field Gel Electrophoresis (PFGE), SCC*mec* typing and Multilocus Sequence Typing (MLST). PFGE revealed major clonal group A, G, L and Q, suggesting dissemination of MRSA in the EMR. Group A harboured mainly SCC*mec* type III and sequence types (STs) ST239 and ST241. The majority of the strains from group G harboured SCC*mec* type I and ST8 and ST247, whereas most strains from group L carried either SCC*mec* type IV or I. Within group L, ST8 and 228 were found, belonging to clonal complexes (CC) 8 and 5, respectively. Most strains from group Q included SCC*mec* type II and were sequence typed as ST225. Both ST225-MRSA-II and ST241-MRSA-III were novel findings in Germany. In addition, the SCC*mec* type of two isolates has not been described previously. One strain was classified as SCC*mec* type III, but harboured the *pls* gene and the *dcs* region. Another strain was characterized as SCC*mec* type IV, but lacked the *dcs* region. In addition, one isolate harboured both SCC*mec* type V and Panton-Valentine leukocidin. Finally, the SCC*mec* type of the strains was found to be correlated with the antibiotic susceptibility pattern.

INTRODUCTION

Staphylococcus aureus is a potentially pathogenic bacterium that can cause various diseases, such as post-operative wound infections and necrotizing pneumonia (21). *S. aureus* has a strong adaptive power to antibiotics. Since the introduction of methicillin in 1959, methicillin-resistant *S. aureus* (MRSA) strains have been isolated, first in the United Kingdom in 1961, and subsequently in other parts of the world. Although most of the MRSA strains are hospital-acquired (HA-MRSA), community-acquired strains (CA-MRSA) have also recently been reported (4).

Resistance of MRSA strains to methicillin is determined by the presence of the *mec*A gene, which encodes the penicillin binding protein (PBP) 2a. The *mec*A gene is localized on a mobile genetic element, which is designated the Staphylococcal Cassette Chromosome *mec* (SCC*mec*) (3, 15, 22). Currently, five main types of SCC*mec* (type I to V) are distinguished. SCC*mec* types I, II and III are associated with HA-MRSA, whereas types IV and V are associated with CA-MRSA (13, 16). SCC*mec* types I, IV or V exclusively encode resistance to β-lactam antibiotics. By contrast,

SCC*mec* types II and III determine multi-resistance as these cassettes carry both integrated plasmid sequences (e.g. pT181 and pUB110) and transposons (e.g. Tn*554*) containing drug resistance genes. Besides the resistance genes on SCC*mec*, *S. aureus* can also carry drug resistance genes on other sites of its chromosome and on plasmids. Also situated on SCC*mec* are genes responsible for the regulation of the transcription of *mec*A: Δ*mec*R1 (on SCC*mec* types I, IV and V), *mec*R1 and *mec*I (on SCC*mec* type II and III) (14, 15, 16). For integration into and excision from the chromosome at a specific site (*attBscc*), genes encoding cassette chromosome recombinases (*ccr*) are located within the SCC*mec* elements. These genes are designated *ccr*A1 and *ccr*B1 (in SCC*mec* type I), *ccr*A2 and *ccr*B2 (in SCC*mec* type II and IV), *ccr*A3 and *ccr*B3 (in SCC*mec* type III) and *ccr*C (in SCC*mec* type V) (7, 13, 16).

The Euregio Meuse-Rhine (EMR) is a region consisting of the Belgian provinces of Limbourg and Liège, the German-speaking region of Belgium, the region Aachen in Germany and the southern part of the Dutch province of Limbourg, with an area of 10.478 km^2. Cross-border patient mobility and free access to health care facilities within the European Union (EU) in general, and the EMR in particular, is an important issue for patients, doctors, hospitals, sickness funds and the health care insurance companies. Each year many thousands of the 3.7 million inhabitants of the EMR cross the border to visit a medical specialist or a hospital on the other side of the border. In an official publication of the European Commission (Eurocommissioner David Byrne, Opening Speech Maastricht Conference on Cross-border Health Care, June 8th 2004, Maastricht, The Netherlands), the EMR was therefore recently mentioned as a model region for the EU in the field of cross-border health care and cross-border co-operation of hospitals and sickness funds. Nevertheless, an important issue of concern that is related to cross-border health care is the dissemination of multi-resistant bacteria. In this regard, it is interesting to note that the three countries forming the EMR differ considerably in the prevalence of MRSA isolated in hospitals (23.6%, 13.8% and 0.6% in Belgium, Germany and The Netherlands, respectively) (32). Consequently, the cross-border transfer of patients may have an important impact on the dissemination and prevalence of MRSA, in particular in cases where patients are transferred from countries with a relatively high prevalence to a country with a low prevalence. Therefore, we investigated the dissemination of MRSA isolates between hospitals from the EMR during the last five years. The MRSA isolates were subjected to Pulsed-Field Gel Electrophoresis (PFGE), SCC*mec* typing, and Multilocus Sequence Typing (MLST) (2). As the presence of Panton-Valentine leukocidin (PVL) genes was suggested to be

an important characteristic of CA-MRSA (33), the isolates were also subjected to a PVL-specific real-time PCR.

MATERIALS AND METHODS

Clinical isolates

One hundred and fifty-two isolates of MRSA from individual patients isolated between December 1999 and February 2004 from five geographically closely related hospitals in the EMR were included in the study (Table 1). These included two Belgium hospitals (Hospital East-Limbourg, Genk, a 822-bed general hospital, and General Hospital Vesalius, Tongeren, a 355-bed general hospital), one German hospital (Universitätsklinikum Aachen, a tertiary 1500-bed university hospital) and two Dutch hospitals (Atrium Medical Centre, Heerlen, a 811-bed general hospital and University Hospital Maastricht, a tertiary 680-bed university hospital). All strains were identified as *S. aureus* by catalase and coagulase testing. Methicillin resistance was determined by the disk diffusion test with oxacillin concentration disks, according to the guidelines of the National Committee for Clinical Laboratory Standards (NCCLS) (23) and by PCR amplification of the *mec*A gene (27).

Reference strains

MRSA strains COL, BK2464, ANS46, HDE288 and WIS were used as *reference strains for SCCmec* type I, II, III, IV and V, respectively (16, 24). *S. aureus* strain 1206 was used as a positive control for the Tn*554* PCR (35). For PFGE, reference strain *S. aureus* Ps 47 was used as a molecular weight marker, whereas MRSA strains BK2464, COL, HDE288, HU25 and PER34 were used as reference strains for the New York/Japan, Archaic, Pediatric, Brazilian and Iberian clonal type, respectively (25).

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed by micro-broth dilution according to the NCCLS guidelines (23) for the following antibiotics: amikacin, amoxicillin, cefazolin, ciprofloxacin, clindamycin, co-trimoxazol, doxycyclin, erythromycin, flucloxacillin, gentamicin, penicillin, rifampicin and vancomycin.

SCCmec typing

SCC*mec* typing was essentially carried out as described by Oliveira *et al* (24) in which *mec*A and six different loci on SCC*mec* (Figure 1) were amplified by PCR with the following modifications. PCR amplification of *mec*A sequences was carried out with primers *mec*A1 and *mec*A2 (Sigma Genosys, The Netherlands), resulting in a PCR product of 527 bp instead of 162 bp (27). PCR was performed in a volume of 50 µl containing 10 µl of a 0.5 McFarland suspension (1.5 x 10^8 CFU/ml) of the MRSA strain, 0.2 mM of each dNTP (Amersham Biosciences, The Netherlands), 1x PCR reaction buffer (Qiagen, The Netherlands), 1.25 U HotStarTaq (Qiagen, The Netherlands) and primers. The primer concentrations used were similar as previously described (24), except for those of the *mec*A primers, which were 0.6 µM for both *mec*A1 and *mec*A2. The amplifications were performed on a GeneAmp PCR System Model 9600 (Applied Biosystems, The Netherlands) with the following programme: 15 min at 94°C, followed by 30 cycles of 30 sec at 94°C, 30 sec at 53°C and 60 sec at 72°C, followed by a post-extension step of 10 min at 72°C. The PCR products were separated on 2% agarose gels in Tris-Acetate-EDTA (TAE) buffer, stained with ethidium bromide and visualized with UV light using a FluorChem™ Imaging System (Alpha Innotech Corporation, The Netherlands).

PCR for ccrAB and ccrC

Most of the primers used for amplification of *ccr*AB and *ccr*C were as described previously (12, 16). Primer β2, however, was replaced by a primer with the following sequence: 5'-ATTGCCTTGATAATAGCCTCT-3' (primer β2a). The following reaction conditions were used: either 1.2 µM of forward primer β2a or 0.4 µM of forward primer γF respectively, 0.4 µM of either reverse primer α2, α3, α4, or γR, 0.2 mM of each dNTP, 1x PCR reaction buffer, 2.5 U of HotStarTaq DNA Polymerase and 10 µl of a 0.5 to 1 McFarland suspension (1.5 to 3 x 10^8 CFU/ml) in a total volume of 50 µl. Amplification was performed on the GeneAmp PCR System Model 9600 using the following programme: 15 min at 95°C, followed by 35 cycles of 1 min at 95°C, 1 min at 55°C and 1 min at 72°C, followed by an extension step of 10 min at 72°C. PCR products were analyzed by electrophoresis through 1% agarose gels as described above.

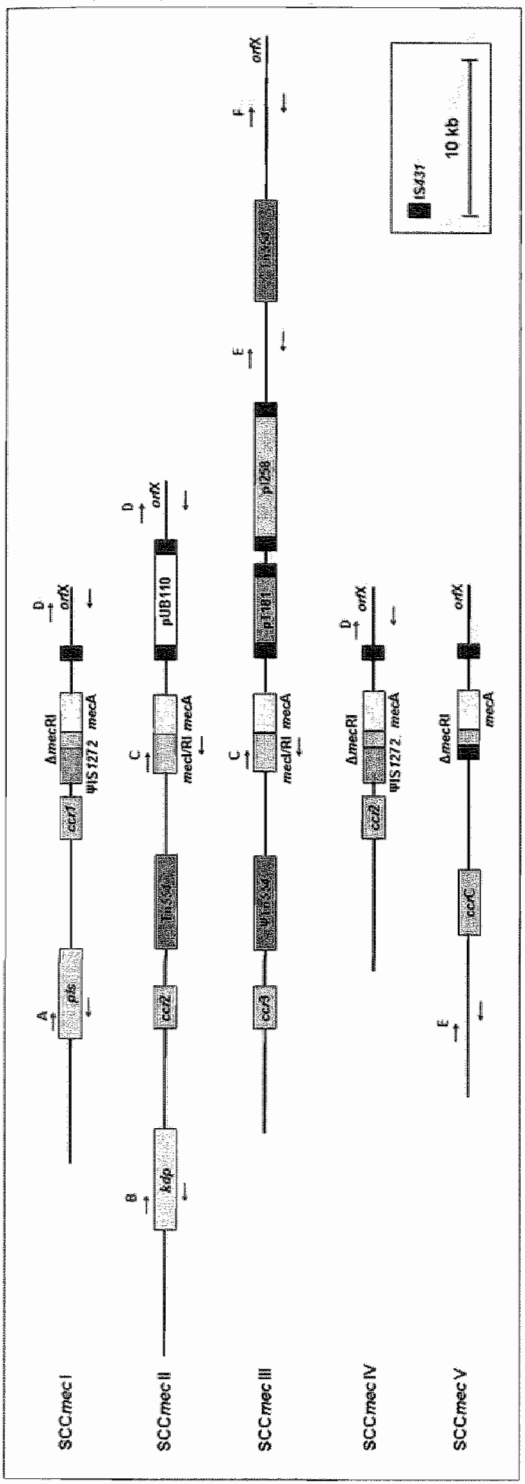

Figure 1. Schematic drawing of SCC*mec* type I to V. The major elements (*ccr* genes, IS*431*, IS*1272*, *mecA*, *mecI*/R1, *orfX*, pI258, pT181, pUB110 and Tn*554*) of the five SCC*mec* types are given as are the six different loci (A to F) used for the typing of SCC*mec* according to the method of Oliveira and de Lencastre (24). The primers of the PCR for the six different SCC*mec* loci are indicated by arrows.

PCR for Tn554

The primers for Tn*554* were used as described previously (17). The reaction conditions used were similar to those for the *ccr* PCR. Amplification was performed on the GeneAmp PCR System Model 9600 using the following programme: 15 min at 94°C, followed by 34 cycles of 1 min at 94°C, 1 min at 63°C and 1 min at 72°C, followed by an extension step of 10 min at 72°C. PCR products were analysed by electrophoresis through 1% agarose gels as described above.

Pulsed-Field Gel Electrophoresis (PFGE)

PFGE was carried out essentially as described previously (12). The banding patters were visualized with UV light using a FluorChem™ Imaging System. Subsequently, the patterns were analysed with Dice comparison and unweighted pair group matching analysis (UPGMA) settings with GelCompar II 3.5 (Applied Maths, Sint-Martens-Latem, Belgium) according to the scheme of Tenover *et al* (31). The position tolerance was set at 2.0% and isolates with a similarity index of 0.80 or more were classified as a clonal group (6, 26).

Multilocus Sequence Typing (MLST)

It has previously been shown that MRSA strains from one major clonal group, as demonstrated by PFGE, have the same Sequence Type (ST), or STs that are related to a single Clonal Complex (CC) (6, 8, 26, 30). Therefore, two representative strains from each of the major clonal groups as obtained through PFGE (6, 8, 26, 30) were used for MLST (10). The primers used for MLST were identical to those described previously (10), with the exception of primers *glpF*-Dn and *gmk*-Up, which were replaced by primers *glpF*-Dna (5'-TGGTAAAATCGCATGTGCAATTC-3') *gmk*-Upa (5'-ATCGTTTTATCAGGACCATC-3'), respectively. The PCR products were sequenced using an ALFexpress II automatic sequencer (Amersham Biosciences, The Netherlands). Finally, the ST was determined using the MLST database (http://www.mlst.net).

Real-time PCR for Panton-Valentine leukocidin (PVL)

PVL was detected with a real-time PCR method as described previously (9).

Statistical analysis

The correlation between the SCC*mec* type and the antibiotic susceptibility pattern was determined with canonical discriminant analyses with the software package SPSS 11.0.1 (SPSS Inc., The Netherlands). Canonical discriminant analyses are used for the investigation of one or more normally distributed interval independent variables (SCC*mec* types) and a categorical dependent variable (susceptibility pattern). This is a multivariate technique that considers the latent dimensions in the independent variables for predicting group membership in the categorical dependent variable.

RESULTS

Distribution of SCCmec types

The SCC*mec* type (Figure 1) could be determined for 148 of the 152 (97.4%) clinical isolates of MRSA. Only four of the five different types of SCC*mec* were found (I to IV; Table 1) with the method described by Oliveira *et al* (24). SCC*mec* types I and IV were predominant with 29% and 34%, respectively, and types II and III were less common with 12% and 23%, respectively. The different SCC*mec* types were not distributed similarly among the three countries from the EMR. In Belgium, SCC*mec* type I and IV predominated, whereas in Germany the most common types were type II and III. In The Netherlands, the most frequently found types were type I, III and IV (Table 1).

The SCC*mec* type of four (DOM068, 083, 114 and 012) of the 152 MRSA isolates (3%) could not be determined according to the method described by Oliveira *et al* (24). DOM068 was found to contain loci A, C, D, E as well as F (Table 2), which is uncommon to the prototypes of the five known SCC*mec* types, as shown in Figure 1. Another isolate, DOM083, was also found to have a unique SCC*mec* organization, possessing loci C, D, E and F (Table 2). The SCC*mec* structure of isolates DOM068 and DOM083 was further characterized by determination of the presence of two other loci from the SCC*mec* cassette, i.e. *ccr* and Tn*554*. Both strains were found to possess Tn*554* as well as the *ccr*AB3 gene, indicating that their SCC*mec* cassettes most strongly resemble the type III cassette (Table 2).

The cassette of another strain, DOM114, was found to contain only a single locus from the six loci that were defined by Oliveira *et al* (24), i.e. locus E (Table 2). Since DOM114 was also found to possess the *ccrC* gene, its SCC*mec* cassette can be classified as type V (Table 2).

Table 1. Distribution of SCC*mec* within MRSA isolates in the Euregio Meuse-Rhine, 1999-2004

	Number (%) of					
Country	SCC*mec* I	SCC*mec* II	SCC*mec* III	SCC*mec* IV	NT[a]	Total
Belgium	24 (60)	2 (5)	1 (2.5)	13 (32.5)	0 (0)	40 (100)
Germany	8 (16)	14 (29)	18 (37)	7 (14)	2 (4)	49 (100)
The Netherlands	12 (19)	2 (3)	16 (25)	31 (49)	2 (3)	63 (100)
Total	44 (29)	18 (12)	35 (23)	51 (34)	4 (3)	152 (100)

[a]Not typeable

The fourth 'non-typeable' isolate, DOM012, lacked all six loci as defined by Oliveira *et al.* (24). However, this strain was found to contain the *ccr*AB2 gene, which is characteristic for both type II and type IV cassettes. Since the DOM012 cassette did not contain Tn*554*, it was classified as type IV (Table 2).

Table 2. Results of non-typeable[b] SCC*mec* within MRSA isolates

Code[a]	Country	Locus[b]	*ccr*	*Tn554*	SCC*mec* type
DOM012	The Netherlands	-	*ccrA2*	-	IV
DOM068	Germany	A, C to F	*ccrA3*	+	III
DOM083	Germany	C to F	*ccrA3*	+	III
DOM114	The Netherlands	E	*ccr*C	-	V

[a]All strains were positive for *mec*A, [b]According to the classification scheme of Oliveira and de Lencastre (24). The distribution of the six different loci among the SCC*mec* types are given in Figure 1.

PFGE analyses

Each of the MRSA strains was subjected to analysis by PFGE. On the basis of the PFGE patterns, a dendrogram was constructed (Figure 2). One of the isolates (DOM152) could not be typed due to repeated difficulties with the isolation of DNA from this strain. A total of 32 clonal groups (A to AF) were distinguished, of which four were major clonal groups (A, G, L and Q). Major clonal group A was closely related to the clonal groups B, C, D and E. Taken together, these groups included 34 of the 152 MRSA isolates (22%). A large majority of these 34 strains (88%), isolated from both Germany and The Netherlands, harboured SCC*mec* type III.

The second major clonal group, group G, contained 26 of the 152 MRSA isolates (17%) of which 21 (81%) harboured SCC*mec* type I, and 5 (19%) harboured SCC*mec* type IV. Most isolates from this group originated from Belgium and The Netherlands. Group G was also found to include reference strain COL, representative for the Archaic clone, which is one of the six major MRSA clones spread worldwide (2). Clonal group F, which was related to group G, comprised only 4 isolates (3%), three of which harboured SCC*mec* type IV and one strain harboured SCC*mec* type V. The reference strain for the Iberian clone (2), PER34, was linked to clonal group F.

The third major clonal group, group L, included 26 of the 152 MRSA isolates (17%). Of these, 18 (69%) contained SCC*mec* type IV, 6 (23%) contained SCC*mec*

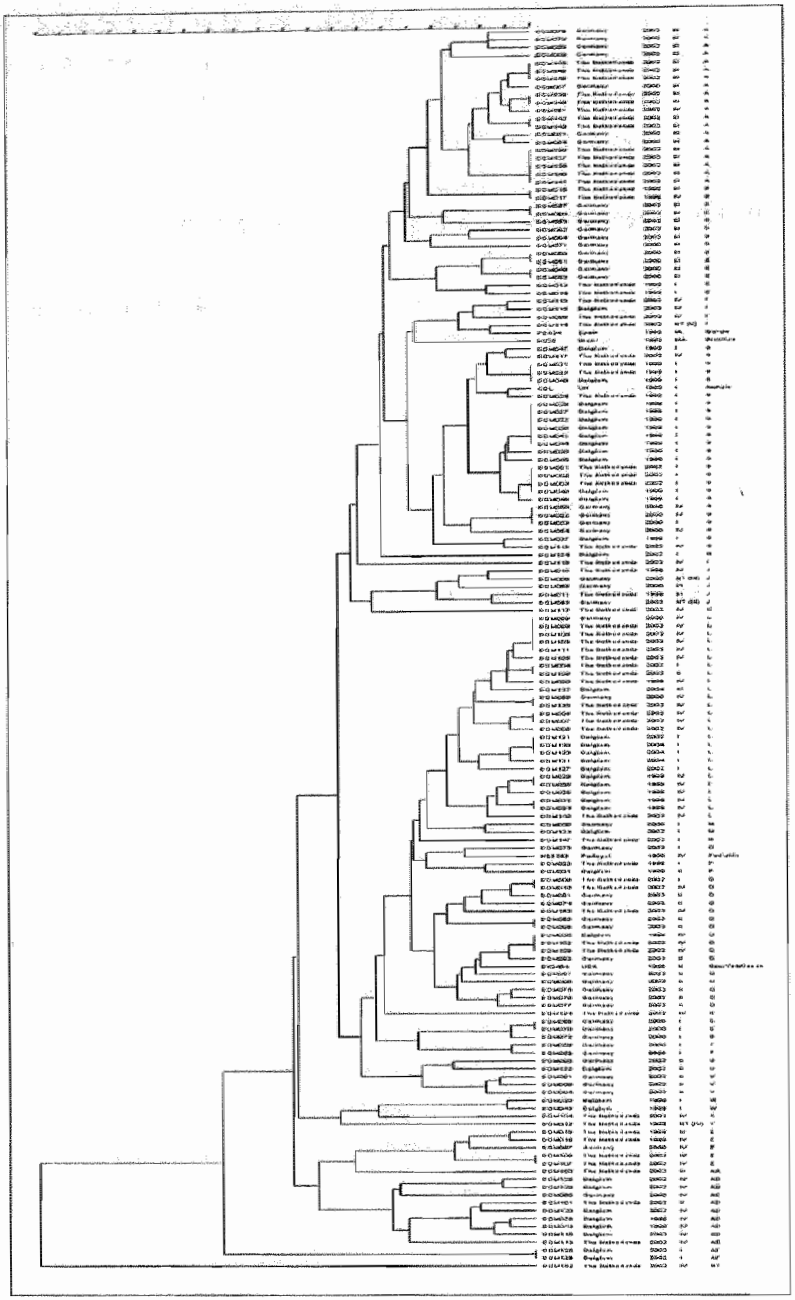

Figure 2. Dendrogram of the 152 clinical MRSA isolates and five reference clones. The five columns on the right represent MRSA isolate code, country of origin, year of isolation, SCC*mec* type and clonal group respectively. NT, Not typeable.

type I, 1 (4%) contained SCC*mec* type II, and 1 (4%) contained SCC*mec* type III. Most of the strains were isolated in Belgium and The Netherlands. None of the reference strains were linked to this clonal group.

The fourth major group, group Q, contained 16 of the 152 MRSA isolates (11%). Ten (63%) of these carried SCC*mec* type II, whereas 5 (31%) contained SCC*mec* IV, and 1 (6%) contained SCC*mec* type I. All except one of the strains from this group was isolated in either Germany or The Netherlands. Group Q also included reference strain BK2464, which is a representative of the New York/Japan clone (2).

MLST analyses

Two representative strains from each major clonal PFGE group were subjected to MLST. As shown in Table 3, MLST identified six different sequence types (ST8, 225, 228, 239, 241, 247) that belonged to two clonal complexes (CC5 and 8). In major clonal group A, two different STs, which both belong to CC5, were found: (i) ST239-MRSA-III, a single locus variant (SLV) of ST8 and representative for the Brazilian clone, and (ii) ST241-MRSA-II, an SLV of ST239-MRSA-III (at locus *yqiL*). The two STs that were found in major clonal group G are different SLVs of a single ST, i.e. ST250; ST247-MRSA-I is an SLV at the *gmk* locus, and ST8-MRSA-I is an SLV at the *yqiL* locus of ST250 (11). Interestingly, the strains from major clonal group L (DOM111 and DOM131) were typed as ST8-MRSA-IV and ST228-MRSA-I, respectively, which belong to different clonal complexes (CC8 and 5, respectively). Both strains from major clonal group Q were typed as ST225-MRSA-II, an SLV at the *tpi* locus of ST5 (11).

Prevalence of PVL

Only two (DOM103 and DOM114) of the 152 MRSA isolates (1.3%) contained PVL. Although both strains were isolated in The Netherlands in 2003, they differed in both SCC*mec* type and PFGE type. Strain DOM103 was classified within major clonal group L, harbouring SCC*mec* type IV, whereas strain DOM114 was classified within group F, carrying SCC*mec* type V (Figure 2).

Correlation between SCCmec type and antibiotics susceptibility pattern

The antibiotic resistance pattern for the SCC*mec* types is presented in Table 4. Only the non β-lactam antibiotics are presented in this table, since all MRSA strains

Table 3. Typing results of the four major clonal groups

Code	Country	Major Clonal Group	SCC*mec* type	MLST	ST[b]	CC[c]
DOM078	Germany	A	III	2-3-1-1-4-4-30	241	8
DOM141	The Netherlands	A	III	2-3-1-1-4-4-3	239	8
DOM038	Belgium	G	I	3-3-1-12-4-4-16	247	8
DOM053	Germany	G	I	3-3-1-1-4-4-3	8	8
DOM111	The Netherlands	L	IV	3-3-1-1-4-4-3	8	8
DOM131	Belgium	L	I	1-4-1-4-12-24-29	228	5
DOM077	Germany	Q	II	1-4-1-4-12-25-10	225	5
DOM092	Germany	Q	II	1-4-1-4-12-25-10	225	5

[a]Sequence Type, [b]Clonal Complex

were resistant to the four β-lactam antibiotics tested, e.g. amoxicillin, cefazolin, flucloxacillin, and penicillin. To investigate if a correlation exists between the SCC*mec* type and the antibiotic susceptibility pattern of MRSA isolates, canonical discriminant analyses were performed. As shown in Figure 3, the SCC*mec* types were centred around the four group centroids, indicating that the SCC*mec* type and the susceptibility pattern were indeed correlated. The antibiotic susceptibility pattern had a predictive value of 84.1% for SCC*mec* type I, 83.3% for SCC*mec* type II, 85.7% for SCC*mec* type III and 86.3% for SCC*mec* type IV. As shown in Table 4 and Figure 3, the correlation is more pronounced for SCC*mec* type II, III and IV than for SCC*mec* type I.

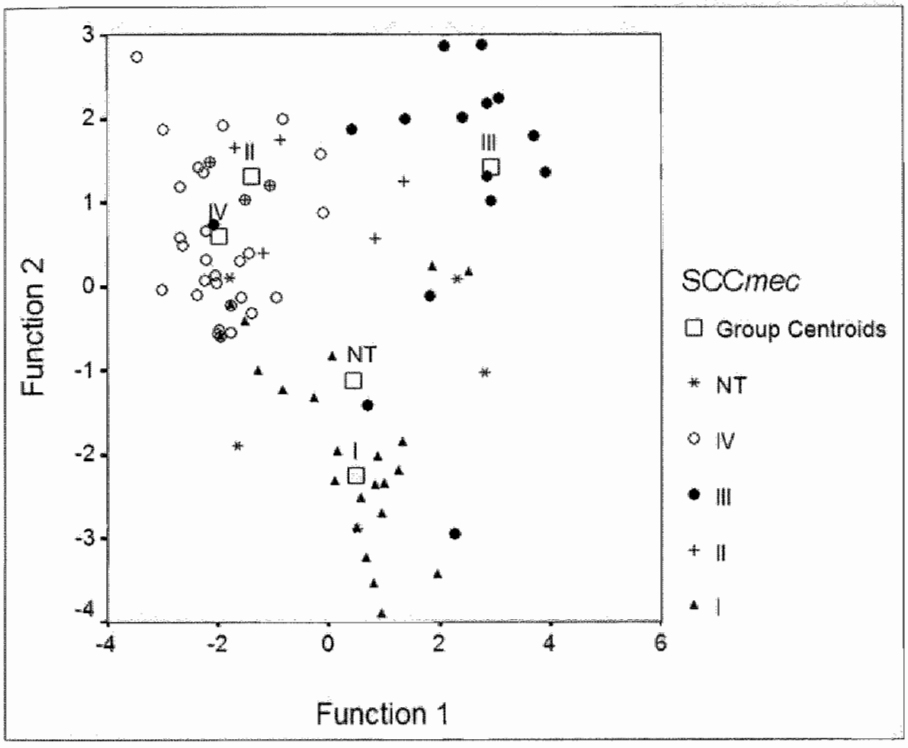

Figure 3. Statistical analyses of the SCC*mec* type and the antibiotic susceptibility pattern. The discriminant function 1 and function 2 are latent variables that are created as a linear combination of discriminating variables. NT, Not typeable.

Table 4. Distribution of the non β-lactam antibiotic resistance pattern among the 152 MRSA strains and their corresponding SCC*mec* types

	Number (%) of resistance MRSA strains								
SCC*mec* type	AMK[a]	CIP[b]	CLI[c]	COT[d]	DOX[e]	ERY[f]	GEN[g]	RIF[h]	VAN[i]
I	43 (98)	42 (96)	32 (73)	42 (96)	17 (39)	38 (86)	42 (96)	7 (16)	0 (0)
II	17 (94)	17 (94)	17 (94)	3 (17)	1 (6)	17 (94)	1 (6)	1 (6)	0 (0)
III	35 (100)	33 (94)	30 (86)	34 (97)	32 (91)	34 (97)	32 (91)	6 (17)	0 (0)
IV	34 (67)	40 (78)	11 (22)	10 (20)	0 (0)	28 (55)	22 (43)	0 (0)	0 (0)
NT[j]	3 (75)	2 (50)	1 (25)	2 (50)	2 (50)	1 (25)	3 (75)	2 (50)	0 (0)

[a]Amikacin, [b]Ciprofloxacin, [c]Clindamycin, [d]Co-trimoxazol, [e]Doxycycline, [f]Erythromycine, [g]Gentamicin, [h]Rifampicin, [i]Vancomycin, [j]Not typeable

Discussion

Monitoring the dissemination of MRSA in the EMR is important, since known and novel MRSA clones may spread from country to country through cross-border patient care. In particular, the spread of MRSA harbouring either SCC*mec* type II or III, which encode multi-resistance, could pose a serious threat to health care facilities.

In this study, 152 MRSA strains were characterized, isolated in hospitals from the EMR between 1999 and 2004. Typing of the strains by PFGE revealed four major clonal groups, suggesting dissemination of MRSA in the EMR. The strains that were classified within major clonal group A and within the closely related, minor clonal groups B to E, comprised 22% of all isolates. Two representative strains from group A were also typed by MLST, and were classified as ST239-MRSA-III and ST241-MRSA-III. Both STs form part of CC8 (11). Although strains with the signature of ST239-MRSA-III were previously found in both Germany and The Netherlands, this study is the first to report the presence of ST241-MRSA-III in Germany (11).

The second major clonal group, group G, included MRSA strains harbouring mainly SCC*mec* type I (Figure 2). MLST of two representative clones from group G revealed two different STs, which were classified within the same clonal complex (CC8), i.e. ST8-MRSA-I and ST247-MRSA-I. Both STs have previously been found in the countries from the EMR: ST8-MRSA-I in Belgium and The Netherlands, and ST247-MRSA-I in Belgium and Germany (8, 11, 36).

MRSA strains from major clonal group L harboured mainly SCC*mec* types IV and I. From this group, two strains with a different SCC*mec* type (IV and I) were selected for analysis by MLST. Thus, these strains were classified as ST8-MRSA-IV and ST228-MRSA-I, respectively. Interestingly, these STs belong to different clonal complexes, i.e. CC8 and CC5, respectively. Although these CCs were more related to each other than they are to other CCs (20), the finding of strains from different MLST clonal complexes within a single PFGE clonal group was novel for *S. aureus*. Nevertheless, this finding was in line with previous reports that demonstrated the higher discriminatory power of MLST over PFGE for bacterial species other than *S. aureus*, such as *Vibrio cholerae*, *Salmonella* and *Listeria monocytogenes* (18, 19, 29). Both ST8-MRSA-IV and ST228-MRSA-I were found previously in the EMR countries: ST8-MRSA-IV in Germany and The Netherlands, and ST228-MRSA-I in Belgium and Germany (11, 36).

MRSA strains from the fourth major clonal group, group Q, harboured mainly SCC*mec* type II. Two representatives from this group were both typed as ST225-MRSA-II, an ST belonging to CC5. Although ST225-MRSA-II has previously been

found in the USA (30), the finding of this ST in Germany is novel. Since ST225-MRSA-II is a single-locus variant (SLV) at the *tpi* locus of strain ST5-MRSA-II, which was previously found in Belgium (8), ST225-MRSA-II may be derived from ST5-MRSA-II. Recent studies reported the finding of ST22-MRSA-IV and ST45-MRSA-IV in Belgium and ST45-MRSA-I and ST45-MRSA-IV in The Netherlands (8, 34). These STs, however, were not found in this study.

From four of the 152 MRSA isolates (3%) the SCC*mec* type could not be determined using the method described by Oliveira *et al* (24). The percentage of non-typeable SCC*mec* cassettes was low compared to other studies, in which 10 to 15% could not be typed (5, 12). Since two of the non-typeable MRSA strains (DOM068 and DOM083) were found to possess the *ccr*AB3 gene, they could be considered to be SCC*mec* type III strains. However, compared to the type III cassette prototype (Figure 1), strain DOM068 contained two additional loci, locus A (*pls* gene) and D (*dcs* region). Strains with a similar organization of *SCCmec* loci as DOM068 have not been reported yet. In contrast, a strain with an additional locus D as opposed to the type III prototype, as seen in strain DOM083, has previously been described by Aires de Sousa and co-workers (1).

Another non-typeable strain, DOM012, was found to contain only a single *SCCmec* locus apart from *mec*A. This locus, *ccr*AB2, was present in the prototype cassettes of both type II and IV. However, since strain DOM012 gene neither possessed *mec*I (locus C) nor Tn*554*, its cassette had a higher similarity with the cassette of type IV than with that of type II. Also the resistance to β-lactam antibiotics found was in line with SCC*mec* type IV. We therefore concluded that strain DOM012 carries a SCC*mec* type IV, but lacks locus D.

The low prevalence of PVL was in accordance with previous studies (9, 28). Both strains were very likely not related as they were classified within a different clonal group and harboured a different SCC*mec* type. DOM114 is to our knowledge the first reported PVL-positive MRSA strain carrying SCC*mec* type V.

Although antibiotic resistance in *S. aureus* can also be determined by sequences other than SCC*mec*, a correlation of approximately 85% was found between the antibiotic susceptibility pattern and the SCC*mec* type. Rapid identification of the SCC*mec* type of MRSA isolates by PCR could therefore be useful to predict the antibiotic susceptibility pattern of isolates and, consequently, guide the choice of antibiotics used for treatment. Hence, the identification of the SCC*mec* type of clinical isolates might contribute to prevent the unnecessary use of vancomycin, which is only needed in case of MRSA isolates harbouring SCC*mec* type II or III.

In summary, MRSA strains belonging to clonal complexes 5 and 8 were disseminated in the EMR and several 'new' types were found: both ST225-MRSA-II and ST241-MRSA-III were novel findings in the German part of the EMR. Furthermore, one strain was classified as SCC*mec* type III, but contained the *pls* gene and the *dcs* region and another strain was characterized as SCC*mec* type IV, but lacked the *dcs* region. One isolate was found harbouring both SCC*mec* type V and PVL.

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CHAPTER 5

The prevalence of the *Staphylococcus aureus tst* gene among community- and hospital-acquired strains and isolates from Wegener's Granulomatosis patients

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ABSTRACT

To allow rapid identification of toxic shock syndrome toxin-1 (TSST-1)-producing *Staphylococcus aureus* strains, a real-time PCR assay for the detection of the *tst* gene, which encodes TSST-1, was developed. The assay was applied to *S. aureus* isolates from patients with Wegener's Granulomatosis (WG), as well as isolates that were classified as either community- (CA) or hospital-acquired (HA). No significant difference in the percentage of *tst*-positive strains was observed between isolates from WG patients and CA isolates (24% and 25%, respectively). In contrast, only 14% of the HA isolates were *tst*-positive ($p<0.05$). Investigation of the clonal relationship between *tst*-positive CA and HA strains could indicated the recent emergence of a virulent *S. aureus* clone in the community.

INTRODUCTION

The pathogenicity of *Staphylococcus aureus* can in part be attributed to the production of pyrogenic toxins. Because secretion of these toxins causes excessive stimulation of T-lymphocytes, they are called superantigens (SAgs) (13). To date, fifteen different SAgs have been identified in *S. aureus*, i.e. staphylococcal enterotoxins (SE) A to E and G to M, exfoliative toxins (ET) A and B, and toxic shock syndrome toxin-1 (TSST-1) (1, 10, 16).

TSST-1 is a 29.1-kDa protein that is encoded by the *S. aureus tst* gene (7, 11). The release of TSST-1 into the bloodstream may give rise to a variety of severe clinical conditions, such as toxic shock syndrome (TSS), sudden infant death syndrome (SIDS), and Kawasaki syndrome. The *tst* gene is present in up to 70% of the *S. aureus* strains isolated from patients with TSS. TSS is characterized by high fever, erythematous rash formation, hypotension and major oxygen involvement, which may lead to multi organ failure. Without appropriate therapy, a lethal shock may develop within 24 hours after the onset of symptoms (6, 7, 12, 13). Although most cases (two thirds) of TSS are associated with tampon use, an increasing number of cases are related to localized infections, surgical complications and insect bites (12). Musser *et al.* showed that the majority of female patients affected with TSS had a single clone of *S. aureus* that was well adapted to colonization in the genital tract (15). TSS is usually treated with proper drainage of surgical wounds, a high dose of a β-lactam antibiotic and γ-globulin (2). Nevertheless, TSS still has a lethality rate of about 30% (7).

The potency of TSST-1 lies in its ability to efficiently induce T-cell proliferation and activation (10,000-fold more efficiently than other antigens). This is due to bridging

the antigen-presenting cells (APC) and T-lymphocytes through binding the major histocompatibility complex (MHC) class II on APCs and specific variable regions on the β-chain of both CD4 and CD8 antigen receptors. Consequently, a T-helper 1 type response is mounted, which results in the massive release of interleukins and cytokines (6). It has previously been suggested that TSST-1 as well as other SAgs may play a role in patients suffering from Wegener's Granulomatosis (WG), a disease in which organs are damaged through inflammation of blood vessels. This finding was based on the observation of an increased incidence of nasal carriage of *S. aureus* in combination with chronic activation of circulating T-cells in WG patients (17).

In order to both control and monitor the spread of *S. aureus* strains producing TSST-1 in the community as well as in the hospital, it is important to be able to rapidly identify these strains. Therefore, a real-time PCR (TaqMan®) assay for the detection of the *S. aureus tst* gene was developed. The assay was used to determine the prevalence of *tst*-positive strains among community-acquired (CA) and hospital-acquired (HA) *S. aureus* bloodstream isolates, and among isolates from nasal swabs from WG patients. Finally, the clonal relation between the TSST-1-positive CA and HA strains as well as all *S. aureus* isolates from WG patients was investigated using Pulsed-Field Gel Electrophoresis (PFGE).

Materials and Methods

Bacterial isolates

One-hundred and six *S. aureus* strains consisting of 55 TSST-1-negative and 51 TSST-1-positive *S. aureus* isolates, which were previously characterized using a conventional *tst*-specific PCR method (9, 10), were used to investigate the characteristics of the *tst* real-time PCR assay. To test for potential cross-reactivity with other staphylococcal species, clinical isolates of *Staphylococcus epidermidis* (n=14), *S. capitis* (n=3), *S. haemolyticus* (n=4), *S. chromogenes* (n=1), *S. cohnii* (n=1), *S. hominis* (n=1), *S. sciuri* (n=1) and *S. warneri* (n=1) were subjected to the TSST-1 real-time PCR. The strains were identified with the API Staph identification system (Biomérieux, Boxtel, The Netherlands).

A random selection of 86 methicillin-susceptible *S. aureus* (MSSA) bloodstream isolates from individual patients in our hospital, a tertiary 680-bed university hospital, was investigated for the presence of the *tst* gene. These strains, isolated between 1999 and 2003, included 51 CA *S. aureus* isolates (isolated within 48 hours after patient admission to the hospital) and 36 HA *S. aureus* isolates (isolated after 72 hours of

admission). In addition, 16 *S. aureus* strains, isolated from nasal swabs in 2004, from WG patients were investigated. Each of these strains was found to be catalase and coagulase positive, confirming the identification of these isolates as *S. aureus*.

Primer and probe design

Primers (Sigma-Genosys, Haverhill, United Kingdom) and TaqMan®-probes (Applied Biosystems [ABI], Nieuwerkerk a/d Ijssel, The Netherlands) were designed based on the published sequences of the *S. aureus*-specific *tst* and *fem*A genes (Table 1), using the computer programme Primer Express 2.0 (ABI, Nieuwerkerk a/d Ijssel, The Netherlands). The specificity of the primer and probe sequences was confirmed by screening sequence databases using BLAST (http://www.ncbi.nlm.nih.gov/blast).

TaqMan assay for femA and TSST-1

A PCR specific for the *S. aureus femA* gene was developed to serve as a positive control for the *tst*-specific PCR. Assay conditions, such as primer and probe concentrations, were optimized according to the guidelines from both the Primer Express 2.0 software programme and the manual (Protocol) of the TaqMan® Universal PCR Master Mix (ABI, Nieuwerkerk a/d Ijssel, The Netherlands). The following reaction conditions were used in the TaqMan® assay: 0.3 µM of *fem*A_FP or TSST-1_FP, 0.3 µM of *fem*A_RP or TSST-1_RP, 100 nM of *fem*A-PR or TSST-1_PR, 1 x TaqMan® Universal PCR Master Mix (ABI, Nieuwerkerk a/d Ijssel, The Netherlands) and 20 µl of purified genomic DNA (isolated from a 1 McFarland (3×10^8 colony forming units [CFU]/ml) suspension using the Wizard® Genomic DNA Purification Kit (Promega, Leiden, The Netherlands) (15)) or 20 µl of a 1:100 diluted 1 McFarland suspension in a total volume of reaction mixture of 50 µl. Amplification was performed on the ABI PRISM 7000 Sequence Detection System using the following programme: 2 min at 50°C, 10 min at 95°C, followed by 42 cycles of 15 sec at 95°C and 60 sec at 60°C.

Pulsed-Field Gel Electrophoresis (PFGE)

PFGE analyses was carried out by digestion of the *S. aureus* chromosomal DNA with *Sma*I (Invitrogen, Breda, The Netherlands) essentially as described previously (8). The PFGE patterns were analyzed with Dice comparison and unweighted pair group matching analysis (UPGMA) settings with GelCompar II 3.5

Table 1. Characteristics of primers and probes for the *fem*A and TSST-1 real-time PCR

Name	Sequence 5' → 3'	Probe label	Accession nr.	Location	Reference
*fem*A_FP[a]	AGAGTTTGGTGCCTTTACAGATAG		X17688	642-665	3
*fem*A_RP[b]	GTCATAGTGGCCAACAGTTTGC			707-687	
*fem*A_PR[c]	TGCCATACAGTCATTTC	MGB, FAM		668-684	
TSST-1_FP[a]	TCATCAGCTAACTCAAATACATGGATT		J02615	537-563	4
TSST-1_RP[b]	TGTGGATCCGTCATTCATTGTT			624-603	
TSST-1_PR[c]	TCCAATAACCACCCGTTTTATCGCTTGAA	TAMRA, FAM		598-570	

[a]Forward primer, [b]Reverse primer, [c]Probe

(Applied Maths, Sint-Martens-Latem, Belgium) according to the scheme of Tenover *et al* (18). The position tolerance was set at 2.0% and isolates with a similarity index of 0.80 or more were classified as a clonal group.

RESULTS AND DISCUSSION

Determination of the detection limit of the tst real-time PCR assay

Initially, the *tst* TaqMan assay was tested using both purified bacterial DNA as well as bacterial suspensions as input material. Since the assay performed similarly using both types of material (data not shown), the assay was optimized by adding bacterial suspensions directly to the reaction mixtures.

To assess the detection limit of the assay, five ten-fold dilutions were made of a 1 McFarland suspension of a *tst*-positive *S. aureus* strain (HT.2004.0349). The number of CFUs in each dilution was determined with an Eddy Jet automatic spiral platter (IUL Instruments, Barcelona, Spain). Then, the bacterial suspensions were tested directly in the real-time PCR assay in three independent runs. As shown in Figure 1(a), the different bacterial suspensions generated mean Ct values ranging from 19.96 to 37.36. The lower detection limit of the assay was approximately 60 CFU per reaction, which corresponded to approximately 3 CFU/µl, i.e. a 10^{-5} dilution of a 1 McFarland suspension. The standard curve derived from the amplification plot (Figure 1(b)) showed a near optimal slope of -3.4, indicating a near optimal reaction efficiency. Furthermore, the standard curve showed that the assay had a broad dynamic range.

Sensitivity and specificity of the assay

To determine the sensitivity and specificity of the assay, known *tst*-positive (n=51) and *tst*–negative (n=55) *S. aureus* isolates were tested. The *tst*-positive strains produced Ct values between 23.34 and 31.83 (mean=26.51, SD=1.37), which was in the range of the *tst*-positive control strain. All *tst*-negative strains did not generate detectable signals (data not shown). As a control for potential inhibition of PCR reactions, all isolates were also tested for the presence of the *S. aureus*-specific *fem*A gene by real-time PCR. As expected, each of these isolates was positive in this assay, with Ct values ranging from 26.01 to 30.05 (mean=27.98, SD=0.94) (data not shown).

The *tst* PCR assay was investigated further by testing a panel (n=26) of staphylococcal species other than *S. aureus*. None of these strains generated positive

results (data not shown) indicating that there was no cross-reactivity with other staphylococcal species.

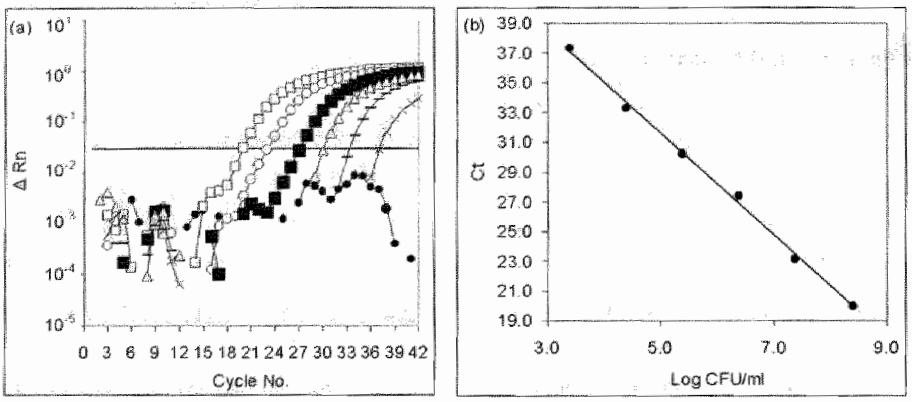

Figure 1. (a) PCR amplification curves of serial dilutions of a *tst*-positive *S. aureus* strain (HT.2004.0349). The strengths of the suspensions ranged from 3 x 10^3 to 3 x 10^8 CFU/ml (1 McFarland). ΔRn indicates the normalized fluorescent reporter value, subtracted from the background value. 3 x 10^8 CFU/ml (-□-); 3 x 10^7 CFU/ml (-o-); 3 x 10^6 CFU/ml (-■-); 3 x 10^5 CFU/ml (-Δ-); 3 x 10^4 CFU/ml (-_-); 3 x 10^3 CFU/ml (-x-); NTC, no template control (-●-); threshold (---). (b) Standard curve generated with the Ct values from the amplification plots shown in (a).

Reproducibility of the assay

The reproducibility of the assay was tested with four *tst*-positive *S. aureus* strains in five independent runs. In these PCR runs, mean Ct values were found of 24.59 (SD=0.45), 26.57 (SD=0.23), 29.37 (SD=0.02) and 26.05 (SD=0.20), respectively. This indicated that the real-time *tst* PCR was highly reproducible.

Prevalence of the tst gene in clinical isolates

Twelve of the 51 (24%) CA *S. aureus* isolates were positive for the *tst* gene and generated Ct values between 25.13 and 34.40 (mean=28.73, SD=3.56). In contrast, only five of 36 (14%) of the HA *S. aureus* isolates were *tst* positive and generated Ct values between 27.02 and 32.11 (mean=29.41, SD=2.17). Four of the 16 (25%) *S. aureus* strains isolated from patients with WG were *tst* positive and generated Ct values between 25.23 and 28.81 (mean=27.08, SD=1.65). This percentage

corresponded to that reported in a previous study, in which a percentage of 19% among WG patients was described (17).

Clonal relationship of tst-positive CA and HA S. aureus and WG clinical isolates

The clonal relationship between the *tst*-positive CA and HA *S. aureus* isolates was studied by PFGE. From the different PFGE patterns a dendrogram was constructed (Figure 2). Six clonal groups (A to F) were found, and the majority (9 out of 17) of the TSST-1-positive *S. aureus* isolates were grouped within major clonal group A. Interestingly, most of these isolates were obtained in 2002 and 2003, and had a CA origin. The other 8 isolates were grouped within five minor clonal groups (B to F). These strains were isolated between 1999 and 2001, and had either a CA or HA origin. The majority of the *tst*-positive CA and HA *S. aureus* strains was resistant to amoxicillin and/or penicillin, but a common resistant phenotype was not found among the CA *S. aureus* strains. In addition, none of the *tst*-positive *S. aureus* strains was positive for PVL (5).

A link between the patients carrying the CA *S. aureus* strains was not found. Some of the patients had been hospitalized during a period of 12 months prior to isolation of the CA *S. aureus* strain. In addition, several patients suffered from different disorders, such as Chronic Obstructive Pulmonary Disease (COPD) and diabetes. Taken together, these data might suggest that during the period 1999-2003, *tst*-positive strains from a single clonal group have become predominant in the community.

The clonal relationship between the *S. aureus* strains isolated from WG patients was investigated with PFGE. One of the strains could not be typed, due to repeated failure to digest DNA with *Sma*I. Seven clonal groups (G to M) were found among the remaining 15 strains. The 4 *tst*-positive *S. aureus* strains were equally distributed between clonal group H and L. However, a relation between clonal groups of *tst*-positive and *tst*-negative *S. aureus* strains was not found (data not shown). Furthermore, the dendrogram showed that only 2 of the strains isolated from WG patients had a clonal relationship with the emerging CA strains (clonal group A). It is assumed that these 2 strains were acquired by the WG patients outside the hospital.

The classification of the *tst*-positive strains from 1999-2003 within 6 different clonal groups is in contrast to the results from Musser *et al.*, who only identified one single clonal group among TSST-1-producing *S. aureus* isolates that caused urogenital TSS [9]. Although the latter strains were typed by multilocus enzyme electrophoresis (MLEE) rather than PFGE, these data may indicate that while clonally unrelated *tst*-

positive strains may be disseminated in the community, a specific disease, such as TSS, may only be caused by specific clonal groups of *S. aureus*.

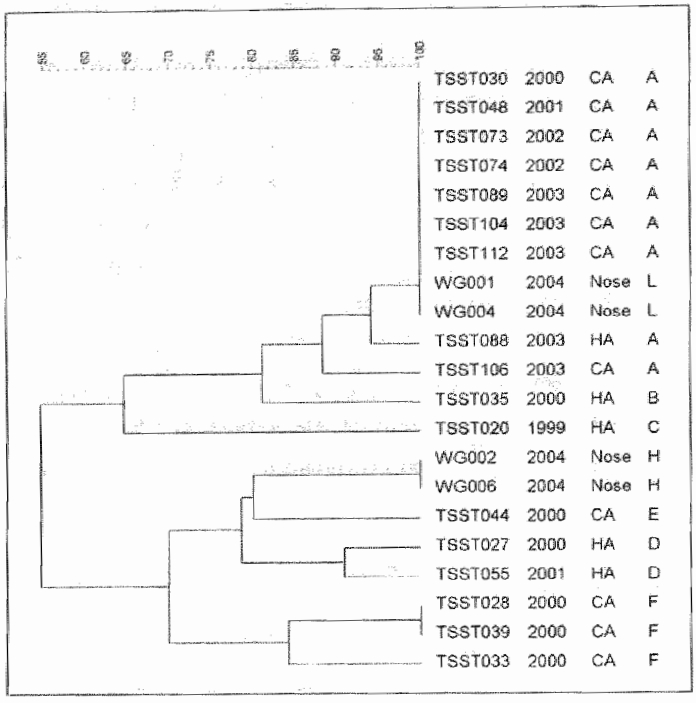

Figure 2. Dendrogram of *S. aureus* isolates (CA, HA and WG) carrying the *tst* gene. The four columns on the right represent isolate code, year of isolation, origin and clonal group, respectively. Community-acquired (CA); hospital-acquired (HA); Wegener's Granulomatosis (WG).

Conclusion

A real-time PCR assay was developed for the detection of the *S. aureus tst* gene. This assay generated results in less than two hours and was, therefore, significantly faster than conventional PCR methods (9 10). The assay was very convenient, since it could be applied directly on diluted bacterial suspensions and previous DNA purification is not required. Furthermore, the assay was found to be highly reproducible, specific as well as robust. The percentage of *tst*-positive strains among *S. aureus* isolates from WG patients was similar to that among isolates from the community. The *tst*-positive CA and HA *S. aureus* strains were grouped in one major (A) and five minor clonal groups (B to F). Since most of the isolates from the major

clonal group were isolated during the later years of the sample period, these isolates may have a more virulent phenotype than isolates which belong to other clonal groups. Furthermore, no relation was found between clonal groups of *tst*-positive and *tst*-negative *S. aureus* strains isolated from WG patients. Since virulent *S. aureus* strains pose an increasing problem worldwide, both in the hospital and in the community, the availability of a rapid, real-time PCR assay for the identification of *tst*-harbouring *S. aureus* strains may contribute to the control of the spread of virulent clones of *S. aureus*.

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CHAPTER 6

Molecular characterisation of methicillin-resistant *Staphylococcus aureus* bloodstream isolates from Croatia

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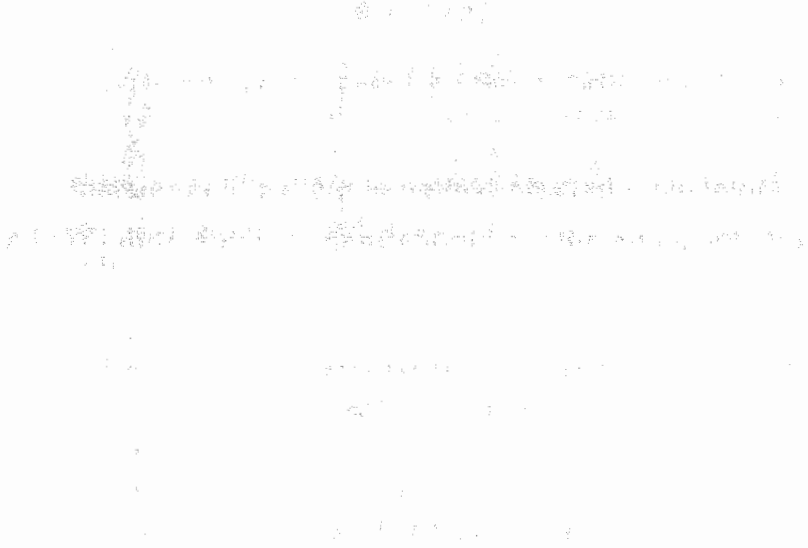

ABSTRACT

A total of 82 hospital-acquired methicillin-resistant *Staphylococcus aureus* (HA-MRSA) bloodstream isolates, collected in 2001 and 2002 in Croatia, were characterised by Pulsed-Field Gel Electrophoresis (PFGE), SCC*mec* typing and Multilocus Sequence Typing (MLST). Furthermore, the presence of the Panton-Valentine leukocidin (PVL) and toxic shock toxin (*tst*) genes was investigated by real-time PCR. All strains were multi-resistant and were distributed among 19 different clonal groups (A to S) as determined by PFGE. Two of the groups, group H and K, harboured the majority of the MRSA strains with 52% and 12%, respectively. The predominant SCC*mec* type that was found among the isolates was type I (89%). Eleven percent of the strains harboured a modified SCC*mec* type III, which, in contrast to the regular type III, contained an additional locus D (*dcs* region). One strain harboured a novel SCC*mec* type, a type V like, due to the presence of the ccrC gene and locus E, in combination with three additional loci (C, D and F). MLST showed the presence of ST111-MRSA-I and ST247-MRSA-I among Croatian MRSA isolates. All isolates were negative for both PVL and TSST-1.

INTRODUCTION

Staphylococcus aureus is recognized as a significant pathogen in postoperative wound infections, pneumonia and urinary tract infections. *S. aureus* has a remarkable ability to rapidly adapt to antibiotic pressure which, over the years, has narrowed the choice of antimicrobial drugs effective against *S. aureus*. Soon after the introduction of methicillin for the treatment of staphylococcal infections, the first methicillin-resistant *S. aureus* (MRSA) strain was isolated in 1961 in the United Kingdom. Since then, a number of MRSA clones have arisen and spread worldwide. MRSA, which was initially a typical nosocomial pathogen, has recently started to appear in the community. It appears that novel strains of MRSA in the community are genetically distinct from MRSA strains originating in the hospital. In some community settings, community-acquired MRSA (CA-MRSA) has become the prevalent form of *S aureus* isolated from cutaneous infections, especially among children. In some hospitals, CA-MRSA is replacing the classic, hospital-acquired MRSA (HA-MRSA) (2, 9, 13, 17, 21).

The resistance of staphylococci to methicillin and all other β–lactam antibiotics is associated with the expression of penicillin-binding protein 2a (PBP2a), which is not present in susceptible staphylococci. This protein is encoded by the *mec*A gene, which is situated on a mobile genetic element, Staphylococcal Cassette Chromosome *mec* (SCC*mec*). SCC*mec* is a genomic island that is inserted at the 3' end of *orf*X and is located near the replication origin of *S. aureus*. Five different SCC*mec* types have so

far been found in MRSA strains. SCC*mec* types I, II and III are found in strains isolated from in-patients, from people with a history of prior hospitalisation, or health care workers (HA-MRSA), while SCC*mec* types IV and V are mainly associated with CA-MRSA. SCC*mec* contains the *mec* complex (*mec*A and its regulators) and the *ccr* gene complex, which encodes site-specific recombinases, responsible for the mobility of SCC*mec*. Several different *ccr* genes have currently been identified: *ccr*A1 and *ccr*B1 in SCC*mec* type I, *ccr*A2 and *ccr*B2 in SCC*mec* type II and IV, *ccr*A3 and *ccr*B3 in SCC*mec* type III, and *ccr*C in SCC*mec* type V (12, 13, 14, 21).

S. aureus can possess a number of virulence factors. Panton-Valentin leukocidin (PVL) is a potential virulence factor and is predominantly associated with furunculosis, cutaneous abscesses and severe necrotic skin infections (15). PVL, together with SCC*mec* type IV, could be a marker for CA-MRSA (27), although PVL was not found in Australian CA-MRSA strains harbouring SCC*mec* type IV (20). TSST-1 is a 29.1-kDa superantigen (SAg) that is encoded by the *S. aureus tst* gene. The release of TSST-1 into the bloodstream may give rise to a variety of severe clinical conditions, such as toxic shock syndrome (TSS), sudden infant death syndrome (SIDS), and Kawasaki syndrome. TSST-1 is one of the most potent of the SAgs and has probably been responsible for an innumerable number of human deaths in the past. TSST-1 is produced by many MRSA strains, particularly in Japanese hospitals (10 16).

Croatia has a MRSA prevalence of 36.7% among bloodstream isolates (26). Information on the clonal distribution of MRSA in Croatia, however, is not available. Furthermore, nothing is currently known about the prevalence of PVL and TSST-1 among Croatian MSRA isolates. Therefore, the aim of this study was to determine the clonal distribution of MRSA among bloodstream isolates in Croatia isolated in 2001 and 2002 using Pulsed-Field Gel Electrophoresis (PFGE), SCC*mec* typing and Multilocus Sequence Typing (MLST), and to determine the presence of the potential virulence factors PVL and TSST-1.

MATERIALS AND METHODS

Clinical isolates

A total of 82 MRSA bloodstream isolates were collected from 11 hospitals in 8 Croatian cities during 2001 and 2002 as part of the European Antimicrobial Resistance Surveillance System (EARSS) project. All strains were hospital-acquired, i.e. isolated ≥72 hours after patient admission to the hospital. Reference strains *S. aureus*

ATCC 25923 for PFGE, MRSA COL (SCC*mec* I), BK2464 (SCC*mec* II), ANS46 (SCC*mec* III), HDE288 (SCC*mec* IV) and WIS (SCC*mec* V) for SCCmec typing were used. MRSA Cluster 28 was used as a reference strain for PVL and *S. aureus* HT.2004.0349 as the reference strain for TSST-1. All strains were identified as *S. aureus* by catalase, coagulase and DNAse production.

Susceptibility testing

The MIC of oxacillin was determined by the broth micro-dilution method, according to the guidelines of the National Committee for Clinical Laboratory Standards (NCCLS) (19). Susceptibility testing of the other agents was performed by the disc diffusion on Mueller-Hinton agar plates, according to the NCCLS guidelines (19). The following antimicrobial drugs were tested: penicillin, clindamycin, erythromycin, azithromycin, gentamicin, vancomycin, rifampicin, ciprofloxacin, norfloxacin, trimethoprim, tetracycline, linezolid, chloramphenicol, trimethoprim-sulfamatoxazole and nitrofurantoin (Becton Dickinson, France).

SCCmec typing

SCC*mec* typing was essentially carried out as described by Oliveira *et al* (22) in which *mec*A and six different loci on SCC*mec* were amplified by PCR with the following modifications. PCR amplification of *mec*A sequences was carried out with primers *mec*A1 and *mec*A2 (Sigma Genosys, The Netherlands), resulting in a PCR product of 527 bp instead of 162 bp (24). PCR was performed in a volume of 50 µl containing 10 µl of a 0.5 McFarland suspension (1.5 x 10^8 CFU/ml) of the MRSA strain, 0.2 mM of each dNTP (Amersham Biosciences, The Netherlands), 1 x PCR reaction buffer (Qiagen, The Netherlands), 1.25 U HotStarTaq (Qiagen, The Netherlands) and primers. The primer concentrations used were similar as previously described (22), except for those of the *mec*A primers, which were 0.6 µM for both *mec*A1 and *mec*A2. The amplifications were performed on a GeneAmp PCR System Model 9600 (Applied Biosystems, The Netherlands) with the following programme: 15 min at 94°C, followed by 30 cycles of 30 sec at 94°C, 30 sec at 53°C and 60 sec at 72°C, followed by a post-extension step of 10 min at 72°C. The PCR products were separated on 2% agarose gels in Tris-Acetate-EDTA (TAE) buffer, stained with ethidium bromide and visualized with UV light using a FluorChem™ Imaging System (Alpha Innotech Corporation, The Netherlands).

PCR for ccrAB3 and ccrC

Most of the primers used for amplification of *ccr*AB3 and *ccr*C were as described previously (11, 14). Primer β2, however, was replaced by a primer with the following sequence: 5'-ATTGCCTTGATAATAGCCTCT-3' (primer β2a). The following reaction conditions were used: either 1.2 µM of forward primer β2a or 0.4 µM of forward primer γF respectively, 0.4 µM of either reverse primer α4, or γR, 0.2 mM of each dNTP, 1× PCR reaction buffer, 2.5 U of HotStarTaq DNA Polymerase and 10 µl of a 0.5 to 1 McFarland suspension (1.5 to 3 x 10^8 CFU/ml) in a total volume of 50 µl. Amplification was performed on the GeneAmp PCR System Model 9600 using the following programme: 15 min at 95°C, followed by 35 cycles of 1 min at 95°C, 1 min at 55°C and 1 min at 72°C, followed by an extension step of 10 min at 72°C. PCR products were analyzed by electrophoresis through 1% agarose gels as described above.

PFGE analyses

PFGE was essentially performed as previously described (11). Whole bacterial DNA was prepared and digested in agarose plugs with *Sma*I. Restriction fragments were resolved using CHEF DR II apparatus (Bio-Rad Laboratories, Hemel Hempstead, UK) with the following settings: voltage 6V/cm, temperature 14°C, initial time 5 sec, final time 40 sec and duration 20 hours. The relatedness of the strains was determined with GelCompar II 3.5 (Applied Maths, Belgium) according to the scheme of Tenover *et al* (25). Isolates with a similarity index of 0.80 or more were classified as a clonal group (3, 23).

Multilocus Sequence Typing (MLST)

Two representative strains from the major clonal groups as obtained through PFGE (3, 5, 23) were used for MLST (8). The primers used for MLST were identical to those described previously (8), with the exception of primers *glpF*-Dn and *gmk*-Up, which were replaced by primers *glpF*-Dna (5'-TGGTAAAATCGCATGTGCAATTC-3') *gmk*-Upa (5'-ATCGTTTTATCAGGACCATC-3'), respectively. The PCR products were sequenced using an ALFexpress II automatic sequencer (Amersham Biosciences, The Netherlands). Finally, the sequence type (ST) was determined using the MLST database (http://www.mlst.net).

Detection of PVL and TSST-1

The presence of PVL and TSST-1 was investigated with real-time PCR as described previously (6, 7).

RESULTS

Susceptibility patterns

The 82 MRSA isolates had MIC values for oxacillin of ≥128 μg/ml. All strains were resistant to gentamicin and ciprofloxacin and were susceptible to vancomycin, linezolid and nitrofurantoin. Most of the strains (96%) were resistant to clindamycin, erythromycin and azithromycin. In addition, 36% percent of the strains were resistant to rifampicin, 32% was resistant to tetracycline, 9% was resistant to chloramphenicol and 0.2% was resistant to trimethoprim-sulfamatoxazole.

SCCmec typing

A total of 73 of the 82 (89%) MRSA strains were found to contain SCC*mec* type I. From 9 (11%) strains, the SCC*mec* type was not typeable with the method of Oliveira *et al.* (22). The SCC*mec* of each of these strains was found to harbour *mec*A and loci C, D, E and F. Therefore, these cassettes only differ from SCCmec type III in the presence of locus D. To investigate the nature of these cassettes in more detail, the *ccr* genes of these strains were characterised. In 8 of the 9 MRSA strains, the *ccr* gene was identified as *ccr*AB3. Consequently, these strains were classified as harbouring SCC*mec* type III. The other non-typeable MRSA strain (79) harboured *ccr*C, which is normally only carried on SCC*mec* type V.

PFGE analyses

PFGE analyses were performed on the MRSA strains and a dendrogram was constructed (Figure 1). Three of the 82 MRSA isolates could not be typed, either due to difficulties with the lyses of the bacteria or the subsequent digestion of the DNA. A total of 19 clonal groups (A to S) were found and two of these groups, H and K, were classified as major clonal groups. Clonal group H harboured 43 of the 82 (52%) MRSA

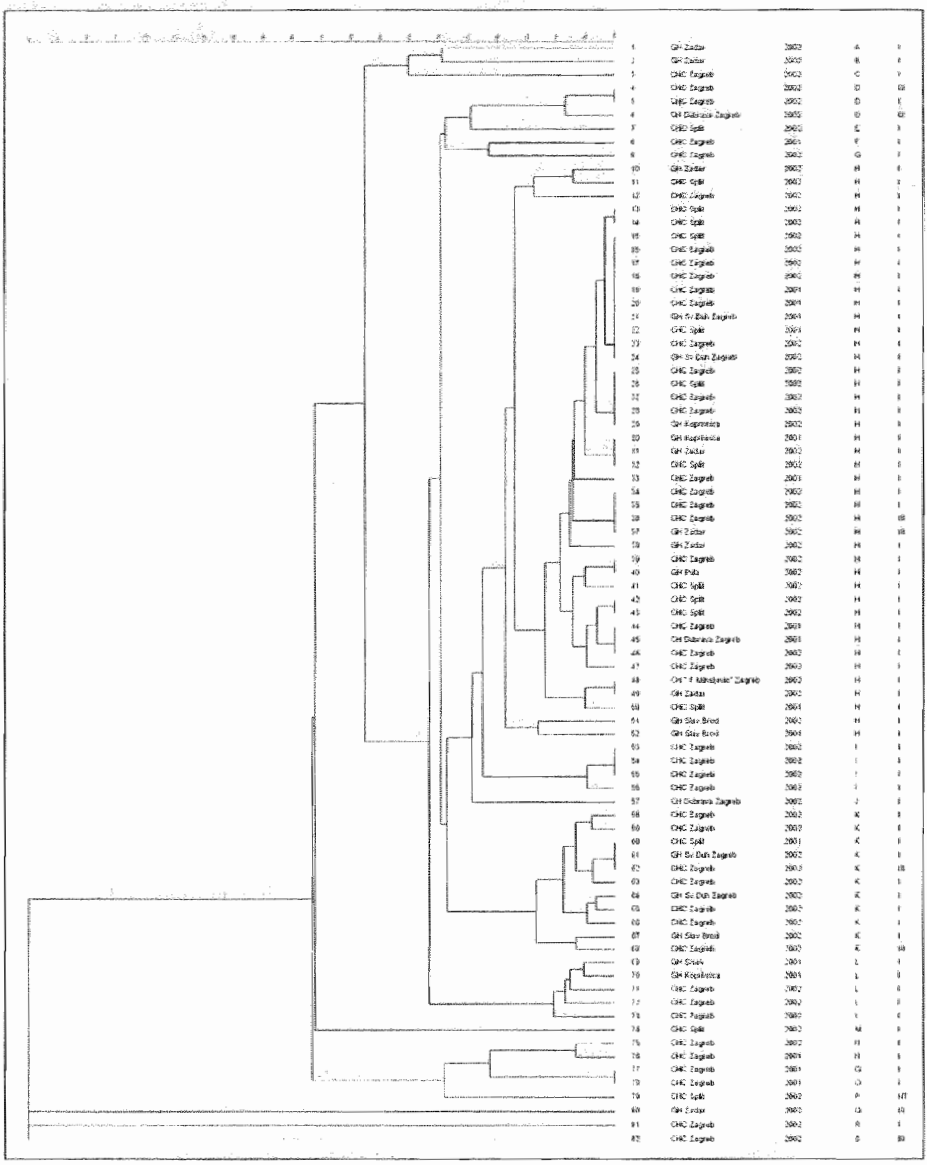

Figure 1. Dendrogram of the 82 MRSA isolates. The five columns on the right represent MRSA isolate code, center, year of isolation, clonal group and SCC*mec* type, respectively. NT, Not typeable.

isolates, whereas clonal group K harboured 11 of the 82 (13%) of the MRSA isolates. In both group H and group K, SCC*mec* type I was predominant at 95% and 82%, respectively. Furthermore, the MRSA strains from groups H and K had a different susceptibility pattern. The majority (91%) of the strains from group H were resistant to rifampicin and tetracycline, while the majority of the strains from group K were susceptible to these antibiotics (84% and 91%, respectively). Strains harbouring SCC*mec* type III were classified in various clonal groups, i.e. group D (2), H (2), K (2), Q (1) and S (1). The results from the SCC*mec* typing and the PFGE analyses indicated that the distribution of particular MRSA genotypes is not restricted to specific hospitals or cities in Croatia.

MLST analyses

Two representative strains from the major clonal groups H and K were characterised by MLST. As shown in Table 1, both strains from group H were identified as ST111, a single locus variant (SLV) of ST228 at the *pta* locus, whereas both strains from group K were classified as ST247.

Table 1. MLST typing results of major clonal group H and K

Code	Major clonal group	SCC*mec* type	MLST profile	ST[a]
13	H	I	1-4-1-4-46-24-29	111
46	H	I	1-4-1-4-46-24-29	111
63	K	I	3-3-1-12-4-4-16	247
65	K	I	3-3-1-12-4-4-16	247

[a]Sequence type

PVL and TSST-1 analyses

All 82 MRSA strains were negative for both PVL and the *tst* gene.

Discussion

In this study, the clonal distribution of MRSA isolates in Croatia was described. The collection of strains studied was uniform, since it contains only bloodstream

isolates. Most of the isolates (89%) were found to possess SCC*mec* type I. Eight of the nine strains that did not contain SCC*mec* type I, carried a modified SCC*mec* type III. In contrast to a normal type III cassette, the cassette of these strains harboured locus D, which is usually only found in SCC*mec* type I, II and IV (22). An MRSA strain with a similar SCC*mec* element (HSA10) has previously also been described by Aires de Sousa and co-workers (1).

The single remaining 'non-SCC*mec* type I' strain from this study (strain 79) was found to harbour *ccr*C, together with loci C (*mec*I, normally found in SCC*mec* II and III), D, E (a locus between pI258 and Tn*554*, which is specific for SCC*mec* type III) and F (a locus between Tn*554* and *orf*X, which is specific for SCC*mec* type III) (22). Since *ccr*C is usually only found in combination with locus E in SCC*mec* type V, MRSA strain 79 is likely to harbour a novel SCC*mec* element. Further investigation into the exact structure of this element is required. Evidence for transmission of methicillin resistance between different *S. aureus* strains during therapy suggests that SCC*mec* may be transferable *in vivo* between different strains (28). An encounter of two *S. aureus* strains harbouring different SCC*mec* cassettes could have led to formation of a novel, not previously described, SCC*mec* type, probably through homologues recombination (12).

Typing of the MRSA strains by PFGE revealed 19 clonal groups (A to S), two of which, H and K, were classified as major clonal groups. These results, together with the SCC*mec* typing, indicated that the distribution of particular MRSA genotypes was not restricted to specific hospitals or cities in Croatia. A total of 52% of the MRSA strains were classified within major clonal group H and 95% of these strains harboured SCC*mec* type I. Two representative strains from group H were typed by MLST, and were both classified as ST111-MRSA-I, a SLV of ST228 at the *pta* locus. Previously, only two MRSA isolates were typed as ST111: (i) a strain with an unknown SCC*mec* type (AB-903627/02) from Norway (http://www.mlst.net), and (ii) a strain harbouring SCC*mec* type I from the Czech Republic (18). The notion that ST111-MRSA-I is found in different European countries and not in other parts of the world might indicate that this type is disseminating in Europe.

The second major clonal group, group K, contained 13% of the MRSA isolates; 82% of the strains from this group harboured SCC*mec* type I. Two representative clones from group K were both classified by MLST as ST247-MRSA-I, which is identical to the sequence type of the Iberian clone. This is one of the major MRSA clones currently isolated in European countries, such as the Czech Republic, Germany, Italy, Poland, Slovenia and Switzerland. ST247 is classified in clonal complex (CC) 8 (2, 9).

The *S. aureus tst* gene and PVL were not detected in any of the isolates investigated. Similarly, a previous study reported only a single PVL-positive isolate among 86 bloodstream isolates (4).These results supported the observation that PVL is especially found among strains carrying SCCmec type IV causing severe skin infections (27). Furthermore, the absence of these genes suggested that they are not related to bloodstream infections in Croatia.

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CHAPTER 7

Ciprofloxacin resistance is not a marker for methicillin-resistant *Staphylococcus aureus* in The Netherlands

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ABSTRACT

The objective of this study was to investigate the relationship between ciprofloxacin resistance and methicillin resistance in *S. aureus* in The Netherlands. Of the 891 *S. aureus* strains isolated from 14 Dutch hospitals between 1995 and 2005, 133 isolates were found to be ciprofloxacin resistant. As only eight of these 133 isolates (6%) were classified as methicillin-resistant *S. aureus* (MRSA) strains, ciprofloxacin resistance of *S. aureus* is not a valuable predictive marker for methicillin resistance in the Netherlands. The MRSA strains harboured various SCC*mec* types and were classified into different clonal groups, indicating the presence of different MRSA clones.

INTRODUCTION

Staphylococcus aureus can cause various diseases. Since the introduction of methicillin in 1959, methicillin-resistant *S. aureus* (MRSA) strains have been isolated in the United Kingdom in 1961 and thereafter in other parts of the world. This illustrated that *S. aureus* possesses a strong adaptive power to antibiotics (5). Resistance to methicillin is mediated through the *mec*A gene, which is situated on a mobile genetic element designated Staphylococcal Cassette Chromosome *mec* (SCC*mec*). To date, five different SCC*mec* types (I to V) have been distinguished. Each SCC*mec* type contains genes that encode for resistance to β-lactam antibiotics, regulatory genes and the cassette chromosome recombinase (*ccr*) genes, which are required for chromosomal integration and excision of the cassettes. The cassettes of type II and III carry, in addition to β-lactam antibiotics resistance genes, non-β-lactam antibiotics resistance genes on integrated plasmids and transposons (5, 6).

It has previously been shown that less than 5% of the methicillin-susceptible *S. aureus* (MSSA) strains and over 95% of the MRSA strains are resistant to ciprofloxacin (2, 4, 12). In addition, it was reported that exposure to ciprofloxacin is a significant risk factor for the isolation of MRSA, but not for the isolation of MSSA (2, 4, 12). In *S. aureus*, ciprofloxacin resistance can be caused by either of three mechanisms: (i) mutations in the DNA topoisomerase IV genes (*grl*A and *grl*B), (ii) mutations in the DNA gyrase genes (*gyr*A and *gyr*B), and (iii) the presence of the NorA efflux pump (10). A limited number of drugs are currently available for the treatment of MRSA colonisation and infections. Mupirocin is commonly used as the drug of first choice for the eradication of MRSA colonisation. Nevertheless, both low- and high-level resistance against mupirocin have emerged among MRSA strains, caused respectively by a mutation in the chromosomal *mup* gene and the plasmid-borne *ileS-2* gene (1).

The objective of this study was to investigate the predictive value of ciprofloxacin resistance of *S. aureus* for the isolation of MRSA in The Netherlands. Furthermore, since mupirocin is commonly used as the drug of first choice for the eradication of MRSA, the prevalence of high-level mupirocin resistance in *S. aureus* strains in The Netherlands was investigated.

MATERIALS AND METHODS

Clinical isolates and reference strains

Between 1995 and 2005, a total of 891 *S. aureus* strains were isolated in 14 university and general hospitals in The Netherlands by the Susceptibility Surveillance Study Group. Of these strains, 133 were found to be resistant to ciprofloxacin. These, as well as 117 randomly selected ciprofloxacin-susceptible *S. aureus* strains, were included in this study. MRSA strains COL, BK2464, ANS46 and HDE288 were used as reference strains for SCC*mec* type I, II, III and IV, respectively (8). All strains were cultivated on 5% sheep blood agar and identified by catalase and coagulase tests.

Susceptibility determinations

MIC values for oxacillin were determined with the E-test (AB Biodisk, Sweden). Suspensions of 0.5 McFarland (1.5×10^8 colony forming units [CFU]/ml) were plated on Mueller-Hinton agar plates and incubated for 18 hours at 37°C. A MIC of ≥ 4 µg/ml was defined as resistant. Ciprofloxacin susceptibility was determined according to NCCLS guidelines and a MIC of ≥ 2 µg/ml was defined as resistant (7).

Identification of MRSA strains and SCC*mec* typing

Strains were identified as MRSA by using a multiplex PCR for the *gp* (the 16S rRNA of Gram-positive bacteria), *nuc* (a *S. aureus* specific gene), *ileS-2* (high-level mupirocin resistance) and *mec*A genes as described previously (1, 3). SCC*mec* typing was performed by investigation of the presence of the *ccr* and *mec*I genes using PCR as described previously (3).

Pulsed-Field Gel Electrophoresis (PFGE)

PFGE analyses were carried out by digestion of the *S. aureus* chromosomal DNA with *Sma*I essentially as described previously (3). The banding patters were visualized with UV light using a FluorChem™ Imaging System (Alpha Innotech Corporation, The Netherlands). Subsequently, the patterns were analysed according to the scheme of Tenover *et al* (11).

RESULTS

Prevalence of MRSA

To identify MRSA strains among the 117 ciprofloxacin-susceptible and all 133 ciprofloxacin-resistant *S. aureus* strains, a *mec*A gene-specific PCR was used. MRSA strains were only found among the ciprofloxacin resistant isolates. The number of MRSA strains identified, however, was rather low (8 out of 133 [6%]). The majority of the ciprofloxacin resistant isolates (94%) as well as all ciprofloxacin-susceptible strains tested were MSSA strains. All 8 MRSA strains were isolated from ICU patients in 1995, 1996 and 1998.

*SCC*mec *typing*

To assess the genetic relationship between the different MRSA isolates, SCC*mec* typing was performed. Four strains harboured SCC*mec* type I, one harboured SCC*mec* type II, and three strains harboured SCC*mec* type IV. Interestingly, the MRSA strains harbouring SCC*mec* type I had an MIC for oxacillin of >256 µg/ml, while the MRSA strains harbouring SCC*mec* type II or IV had a rather low MIC for oxacillin (8 or 32 µg/ml) (Table 1).

Clonal relationship of MRSA isolates

To further investigate the relationship between the MRSA isolates, these strains were also typed by PFGE. The different PFGE patterns indicated that the MRSA strains could be classified into four different clonal groups (A to D). Strong similarity was only observed between strains MRSA 8, 9 and 10, which were classified as clonal

Table 1. Characteristics of the eight ciprofloxacin resistant MRSA isolates.

	Isolation							
Code	Center	Year	MIX OXA[a] (µg/ml)	MIC CIP[b] (µg/ml)	*ccr* type	*mec*I	SCC*mec* type	PFGE type
MRSA 2	II	1995	> 256	16	1	-	I	A
MRSA 8	X	1995	> 256	32	1	-	I	B
MRSA 9	X	1995	> 256	32	1	-	I	B1
MRSA 10	X	1995	> 256	32	1	-	I	B1
MRSA 17	IX	1996	8	8	2	-	IV	C
MRSA 58	III	1998	32	16	2	+	II	D
MRSA 60	VII	1998	8	8	2	-	IV	C
MRSA 61	VII	1998	8	16	2	-	IV	C

[a]Oxacillin, [b]Ciprofloxacin

group B, and between strains MRSA 17, 60 and 61, which were classified as clonal group C. The strains from clonal group B were isolated in one hospital in 1995 and harboured SCC*mec* type I, whereas the strains in clonal group D were isolated in two different hospitals in 1996 and 1998, respectively and harboured SCC*mec* type IV (Table 1).

Prevalence of high-level mupirocin resistance

The *ileS-2* gene was found in only one ciprofloxacin-resistant MSSA strain, indicating that mupirocin can still be considered as the drug of choice for the eradication of MRSA in The Netherlands.

Discussion

In this study, the prevalence of, and correlation between, methicillin and ciprofloxacin resistance among 891 *S. aureus* isolates in The Netherlands was investigated. Only 15% of the *S. aureus* isolates in The Netherlands were classified as ciprofloxacin resistant strains. MRSA strains were found exclusively among the group of ciprofloxacin-resistant isolates, albeit that the percentage of *mec*A-positive strains in this group was only 6%. These results imply that methicillin resistance may be considered as a predictive marker for ciprofloxacin resistance, whereas ciprofloxacin resistance is not a useful predictive marker for MRSA in The Netherlands. This is in contrast to the results of Schaefler, who investigated 2,833 *S. aureus* strains from hospitals in New York and found that 149 strains (5.3%) were ciprofloxacin resistant of which 140 were MRSA and 9 were MSSA (9). The relatively low percentage (0.9%) of MRSA strains that was found among the total population of 891 *S. aureus* isolates from The Netherlands was in accordance with a recent study by Tiemersma *et al*, who reported an MRSA prevalence of 0.8% (12).

Among the MRSA isolates, two groups of genetically related strains were identified by PFGE. The three isolates that were classified within PFGE group B were all isolated in the same year in a single hospital. The isolates from this group were also similar with regard to their SCC*mec* type (type I), which corroborates the notion that these isolates are closely genetically related. The isolates from the other PFGE group (group C) were obtained from different hospitals in different years. The members from this group were also similar with respect to their SCC*mec* type (type IV), suggesting that these strains were CA-MRSA [1]. The other two MRSA isolates were classified

within separate PFGE groups and these isolates were found to contain SCC*mec* type I and II, respectively. Interestingly, the average MIC values for both ciprofloxacin and oxacillin for MRSA strains harbouring SCC*mec* type I was higher than for MRSA strains harbouring SCC*mec* type IV. This indicates that a correlation may exist between the SCC*mec* type and the MIC values of oxacillin and ciprofloxacin. The notion that several clonal groups were found among the ciprofloxacin-resistant MRSA strains was in accordance with an earlier study (2).

The mechanisms of resistance against fluoroquinolones on the one hand and methicillin on the other differ significantly. Fluoroquinolone resistance generally arises from spontaneous mutations in existing chromosomal genes (10), whereas methicillin resistance results from the acquisition of new genetic material, i.e. the SCC*mec* cassette (5). In light of this difference, the question arises as to why a large proportion of MRSA strains (100% in this study) are ciprofloxacin resistant. A possible explanation is that the possession of a SCC*mec* cassette is accompanied by a higher mutation rate. Consequently, the mutations related to ciprofloxacin resistance could arise more efficiently. It is also possible that most MRSA strains are selected under conditions of multi-drug treatment, such that antibiotic pressure strongly favours the transmission of multi-resistant *S. aureus* strains. Since point mutations can be acquired 'spontaneously', in contrast to the SCC*mec* cassette, it would be more obvious to find multi-drug resistance in strains that have previously acquired the *mec*A gene than in strains that do not contain this gene. Regardless of their exact nature, it is clear that both dissemination of antibiotic resistance and the relation between the different antibiotic resistance mechanisms will remain intriguing subjects of further study.

Finally, as no high-level resistance to mupirocin was found among the MRSA strains analysed, mupirocin can still be considered as the drug of choice for the eradication of MRSA in The Netherlands.

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CHAPTER 8

General discussion & Summary

Algemene discussie & Samenvatting

GENERAL DISCUSSION & SUMMARY

This thesis describes the molecular characterisation of *Staphylococcus aureus* isolates with respect to the resistance to methicillin and the detection of some virulence factors, i.e. Panton-Valentine leukocidin (PVL) and toxic shock syndrome toxin-1 (TSST-1). In addition, the dissemination of methicillin-resistant *S. aureus* (MRSA) between several European countries and within one country was studied.

As community-acquired MRSA (CA-MRSA) often harbour the genes encoding for PVL, a real-time PCR was developed and evaluated to identify PVL-harbouring *S. aureus* strains (**Chapter 3**). In addition, a real-time PCR method was developed and evaluated to detect the *tst* gene, encoding TSST-1, in *S. aureus* strains (**Chapter 5**). TSST-1 positive strains were found among Wegener's Granulomatosis (WG) patients, CA and hospital-acquired (HA) *S. aureus* isolates (24%, 14% and 25%, respectively). Investigation of the clonal relationship between *tst*-positive CA and HA strains with Pulsed-Field Gel Electrophoresis (PFGE) might indicated the recent emergence of a virulent *S. aureus* clone in the community. Early detection of MRSA using ciprofloxacin resistance as a predictive marker was not useful on the collection of *S. aureus* strains tested (**Chapter 7**).

Using several typing techniques, such as PFGE, SCC*mec* typing and Multilocus Sequence Typing (MLST), as well as the real-time PCRs for PVL and TSST-1, MRSA isolates from different European countries, i.e. Belgium, Croatia, Germany and The Netherlands were investigated.

The Euregio Meuse-Rhine (EMR) is formed by the border regions of Belgium, Germany and The Netherlands (**Chapter 4**). PFGE analyses of MRSA isolates from the EMR (n=152) revealed four major clonal groups, A, G, L and Q, suggesting dissemination of MRSA between and also within the countries forming the EMR. Group A harboured mainly SCC*mec* type III and sequence types (STs) 239 and ST241. The majority of the strains from group G harboured SCC*mec* type I and ST8 and ST247, whereas most strains from group L carried either SCC*mec* type IV or I. Within group L, ST8 and ST228 were found, belonging to clonal complexes (CC) 8 and 5, respectively. Most strains from group Q included SCC*mec* type II and were sequence typed as ST225. Both ST225-MRSA-II and ST241-MRSA-III were novel findings in Germany. In addition, the SCC*mec* type of two isolates has not been described previously: one strain was classified as SCC*mec* type III, but harboured the *pls* gene and the *dcs* region, while another strain was characterized as SCC*mec* type IV, but lacked the *dcs* region. Of the two isolates harbouring PVL, one harboured SCC*mec* type IV and the

other SCC*mec* type V, suggesting that these two isolates were CA-MRSA. Furthermore, the SCC*mec* type was highly predictable (approximately 85%) for the antibiotic susceptibility pattern, suggesting that rapid identification of the SCC*mec* type might be useful to predict the susceptibility pattern and guide the choice of antibiotics used for treatment. This could prevent unnecessary use of the 'last resort antibiotic" vancomycin, which is then only needed in case of MRSA isolates harbouring SCC*mec* type II or III.

Chapter 6 describes the clonal distribution of 82 MRSA bloodstream isolates collected in 2001 and 2002 in Croatia. No PVL and TSST-1 positive isolates were found. All strains were multi-resistant and were distributed among 19 clonal groups (A to S) as investigated by PFGE, two of which, H and K, were major clonal groups. This suggests spread between Croatian hospitals. Group H harboured 52%, whereas group K harboured 12% of the MRSA isolates. The majority (89%) of the strains harboured SCC*mec* type I. A non-standard SCC*mec* type III was found in 11% of the isolates. One strain harboured, besides four loci, *ccr*C, which is normally only found in SCC*mec* type V. MLST analyses showed the presence of ST111-MRSA-I and ST247-MRSA-I, also found in the EMR and in other European countries.

Rapid and accurate molecular characterisation of MRSA, based on the SCC*mec* type and the investigation of the genetic background by MLST, is still a labour intensive task. Two methods are described in the literature to investigate the type of SCC*mec*. The first method of Oliveira *et al* is a multiplex PCR method, where *mec*A and six different loci on SCC*mec* are amplified with PCR (8). The second method by Ito *et al* (4, 5) characterised the SCC*mec* type based on the combination of the *mec* complex and the *ccr* genes. Most studies use the first method, whereas the method of Ito *et al* is now used when the SCC*mec* type cannot be determined with the method of Oliveira *et al*. However, the two methods gave different results when typing SCC*mec* (10). A general and international agreement should be reached to define known and new SCC*mec* types, and one method should be developed and used in future studies. This method should be evaluated on all major MRSA clones as investigated by MLST (2). Since MLST is very labour intensive, a novel molecular typing method for the investigation of the dissemination of MRSA should be developed, that brings with it the speed of *spa* typing and the accuracy of MLST. This method should ideally be a combination of MLST, *spa* typing, SCC*mec* typing and should also include the determination of *S. aureus* specific genes, such as *fem*A, and several important virulence factors, like PVL and TSST-1.

The worldwide emerge of CA-MRSA is a threat to both the community and the hospital environment, since these strains are known to be more virulent than HA-MRSA strains. Further studies should foremost focus on one clear definition of CA-MRSA, instead of the (at least) eight definitions that are now being used (6, 9). Furthermore, studies to investigate the prevalence of CA-MRSA, their genetic background, the presence of a larger panel of virulence factors in CA-MRSA strains and the risk factors for CA-MRSA colonisation should be started. MLST analyses are necessary to investigate if these strains have a ST1, ST30 or ST80 background, the common CA-MRSA background found in the United States of America, Australia and Europe respectively (11). Another question concerning CA-MRSA is the possible relation between SCC*mec* type IV (and V) and PVL, since reports are conflicting as to whether there is a relation between SCC*mec* type IV and PVL (1, 7, 11). This should be performed with a real-time PCR assay, combining the *S. aureus* specific *fem*A gene, the resistance gene *mec*A, *ccr*AB2, *mec*R1 (in combination specific for SCC*mec* type IV) and PVL. CA-MRSA strains with a different genetic background as investigated by MLST should be studied (2, 11).

There are still a number of questions unanswered concerning the molecular evolution of MRSA. One of the most intriguing questions is the origin of SCC*mec*. In this respect, it is noteworthy that Hanssen *et al* studied 39 methicillin-resistant coagulase-negative staphylococci (MRCNS), and 22 had a novel SCC*mec* type (3). These novel SCC*mec* types could give us more information about the possible transfer of SCC*mec* between CNS species and *S. aureus*. Moreover, other studies found novel SCC*mec* types, or SCC elements without *mec*A, which could be a reservoir for antibiotic resistance islands, in *S. aureus*. Further studies should also investigate the role of pets and farm animals in the transfer of SCC*mec*, i.e. is SCC*mec* formed in animals in MRCNS or MRSA and transferred to humans or are humans the source of CNS or *S. aureus* strains carrying SCC*mec*?

It has been suggested that MRSA is more pathogenic than MSSA, but little evidence has been brought forward, except that CA-MRSA, harbouring SCC*mec* type IV and PVL, have caused necrotising pneumonia in young adults. However, a relation between resistance and virulence cannot be ruled out, since it has been shown that sub-inhibitory concentrations of antibiotics influence the expression of virulence factors.

Another question is why a large proportion of MRSA strains are ciprofloxacin resistant. The mechanisms of resistance against fluoroquinolones and methicillin differ significantly. Fluoroquinolone resistance generally arises from spontaneous mutations in existing chromosomal genes, whereas methicillin resistance results from the

acquisition of new genetic material, i.e. SCC*mec*. It is possible that most MRSA strains are selected under conditions of multi-drug treatment, and that antibiotic pressure strongly favours the transmission of multi-resistant *S. aureus* strains. Regardless of the exact nature, it is clear that the dissemination of antibiotic resistance strains will remain an important subject of further studies.

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ALGEMENE DISCUSSIE & SAMENVATTING

Dit proefschrift beschrijft de moleculaire karakterisering van *Staphylococcus aureus* isolaten betreffende de resistentie voor methicilline en de detectie van de virulentie factoren Panton-Valentine leukocidine (PVL) en toxic shock syndrome toxin-1 (TSST-1). Verder is de verspreiding van methicilline-resistente *S. aureus* (MRSA) isolaten in en tussen verschillende Europese landen onderzocht.

Omdat community-acquired MRSA (CA-MRSA) stammen vaak de genen bezitten welke coderen voor het PVL werd een real-time PCR assay ontwikkeld en geëvalueerd voor de identificatie van PVL-producerende *S. aureus* isolaten (**Hoofdstuk 3**). Daarnaast werd een real-time PCR ontwikkeld en geëvalueerd voor de detectie van het *tst* gen, dat codeert voor TSST-1, in *S. aureus* stammen (**Hoofdstuk 5**). TSST-1-positieve *S. aureus* stammen werden gevonden in patiënten met Wegener's Granulomatosis, CA en hospital-acquired (HA) *S. aureus* isolaten (24 %, 14 % en 25 %, respectievelijk). De relatie tussen de *tst*-positieve CA isolaten werd met behulp van Pulsed-Field Gel Electrophoresis (PFGE) onderzocht. Deze resultaten suggereren de verspreiding van een virulente *S. aureus* kloon in de open populatie. Ciprofloxacine resistentie als een voorspellende marker voor de detectie van MRSA was niet bruikbaar op de collectie *S. aureus* stammen welke zijn bestudeerd (**Hoofdstuk 7**).

Om de verspreiding in België, Duitsland, Kroatië en Nederland en tussen de landen onderling te onderzoeken werden MRSA isolaten gekarakteriseerd met behulp van PFGE en Multilocus Sequence Typing (MLST). Tevens werd het SCC*mec* type bepaald en werd de aanwezigheid van PVL en TSST-1 genen onderzocht.

De Euregio Maas-Rijn (EMR) wordt gevormd door de grensstreek van België, Duitsland en Nederland (**Hoofdstuk 4**). MRSA isolaten (n=152) afkomstig uit deze regio werden getypeerd met behulp van PFGE. Vier grote klonale groepen, A, G, L en Q, werden gevonden, hetgeen suggereert dat verschillende MRSA klonen zijn verspreid in de EMR. Groep A werd hoofdzakelijk gekenmerkt door de aanwezigheid van het SCC*mec* type III en sequence type (ST) 239 en ST241. SCC*mec* type I en ST8/ST247 waren aanwezig in de meerderheid van de stammen van groep G, terwijl de meeste stammen van groep L werden gekenmerkt door het SCC*mec* type IV of I. Binnen deze laatste groep werden ST8 en ST228 aangetoond, te onderscheiden in twee verschillende klonale komplexen (CC): CC8 en CC5. Het merendeel van de stammen van groep Q werd gekenmerkt door het SCC*mec* type II en ST225. Zowel ST225-MRSA-II en ST241-MRSA-III zijn nieuwe MRSA typen in Duitsland. Verder

werd in twee MRSA isolaten een nieuw SCC*mec* type gevonden. Eén stam bezat het SCC*mec* type III, met daarnaast het *pls* gen en de *dcs* regio, terwijl de tweede MRSA stam werd gekarateriseerd als het SCC*mec* type IV zonder de *dcs* regio. Eén MRSA isolaat met het SCC*mec* type V was ook positief voor het PVL. Tenslotte werd een correlatie van ongeveer 85 % gevonden tussen het SCC*mec* type en het antibiotica gevoeligheidspatroon van de MRSA stammen. Snelle identificatie van het SCC*mec* type lijkt bruikbaar om het antibiotica gevoeligheidspatroon te voorspellen en zou gebruikt kunnen worden voor de keuze van de juiste antibiotica therapie. Dit kan leiden tot een reductie van het gebruik van vancomycine, dat alleen nodig is voor de behandeling van MRSA isolaten met het SCC*mec* type II of III.

Hoofdstuk 6 beschrijft een studie waarin de genetische achtergrond van 82 MRSA bloedkweek isolaten afkomstig uit Kroatië en geïsoleerd in 2001 en 2002 werd onderzocht. De MRSA stammen waren PVL en TSST-1 negatief. Alle stammen waren multi-resistent en konden met behulp van PFGE worden verdeeld in 19 verschillende klonale groepen (A t/m S). Groep H en K bevatten de meerderheid (52 % en 12 %, respectievelijk) van de MRSA stammen. Verspreiding van MRSA stammen tussen Kroatische ziekenhuizen lijkt dus waarschijnlijk. Het meest voorkomende SCC*mec* type bij deze isolaten was het type I (89 %), terwijl bij 11 % van de stammen een niet-standaard SCC*mec* type III werd gevonden. Deze bezat een extra locus D vergeleken met het SCC*mec* type III prototype. Eén stam bezat een nieuw SCC*mec* type, welke werd gekenmerkt door het *ccr*C gen en locus E, beide karakteristiek voor het SCC*mec* type V, in combinatie met drie extra loci (C, D en F). Verder onderzoek met MLST onderscheidde twee STs, te weten ST111 en ST247.

Snelle en accurate moleculaire karakterisering van MRSA, gebaseerd op het SCC*mec* type en de genetische achtergrond zoals onderzocht met MLST, is nog steeds zeer arbeidsintensief. Twee methoden om het SCC*mec* type te bepalen zijn beschreven in de literatuur. De eerste methode, ontwikkeld door Oliveira *et al*, is een multiplex PCR, waarbij het *mec*A gen en zes verschillende loci op het SCC*mec* worden gedetecteerd. De tweede methode, ontwikkeld door Ito *et al*, karakteriseert het SCC*mec* type gebaseerd op de combinatie van het *mec* complex en de *ccr* genen. De meeste moleculair epidemiologische studies gebruiken de eerste methode en de methode van Ito *et al* wordt tegenwoordig gebruikt indien het SCC*mec* type met de methode van Oliveira *et al* niet kan worden bepaald. Deze twee methoden kunnen echter verschillende resultaten geven bij SCC*mec* typering. Een algemene en internationale erkende definitie voor SCC*mec* dient te worden afgesproken en

vervolgens zou een nieuwe methode ontwikkeld moeten worden voor de typering van bekende en nieuwe SCC*mec* typen. Deze methode zou geëvalueerd kunnen worden op alle bekende MRSA klonen, zoals onderzocht met MLST, en vervolgens kan deze methode in toekomstige studies gebruikt worden. Aangezien MLST een zeer arbeidsintensieve methode is, is het ontwikkelen van een nieuwe moleculaire typeringsmethode voor onderzoek naar de verspreidng van MRSA ten zeerste gewenst. Deze methode dient de voordelen van *spa* typering (snelheid) en MLST (accuratese) te combineren. Deze methode zou een combinatie kunnen zijn van MLST en SCC*mec* typering, alsmede de detectie van een *S. aureus* specfieke gen, zoals *fem*A, en een aantal virulentie factoren, zoals het PVL.

De wereldwijde toename van de prevalentie van CA-MRSA is een bedreiging binnen en buiten het ziekenhuis, omdat deze stammen virulenter zijn dan HA-MRSA stammen. Allereerst zal internationaal consensus bereikt moeten worden over de definitie van CA-MRSA. Vervolgens kunnen studies worden gestart om de prevalentie van CA-MRSA, de genetische achtergrond van CA-MRSA, de aanwezigheid van een groot aantal virulentie factoren in CA-MRSA stammen en de risicofactoren geassocieerd met CA-MRSA kolonisatie te onderzoeken. MLST analyses zijn bij deze studies noodzakelijk, om te onderzoeken of de stammen een ST1, ST30 of ST80 genetische achtergrond hebben, de typische CA-MRSA genetische achtergrond in respectievelijk de Verenigde Staten van Amerika, Australië en Europa. Een andere vraag is de relatie tussen het SCC*mec* type IV (en V) en het PVL, want de verschillende studies geven geen duidelijk antwoord over de relatie tussen het SCC*mec* type IV en het PVL. Deze studie zou moeten worden verricht met behulp van een real-time PCR methode, welke het *S. aureus* specfiek gen *fem*A, het resistentie gen *mec*A, *ccr*AB2, Δ*mec*R1 (in combinatie specifiek voor het SCC*mec* type IV) en het PVL detecteert. Deze methode dient te worden geëvalueerd met CA-MRSA stammen met een verschillende genetische achtergrond, zoals aangetoond met MLST.

Nog steeds zijn een aantal vragen onbeantwoord betreffende de moleculaire evolutie van MRSA. Eén van de meest interessante vragen is de herkomst van SCC*mec*. Het is opmerkelijk dat Hanssen *et al* 39 methicilline-resistente coagulase-negative staphylococcen (MRCNS) onderzochten, waarvan 22 stammen een onbekend SCC*mec* type hadden. Deze onbekende SCC*mec* typen kunnen ons meer informatie verschaffen over de mogelijke overdracht van SCC*mec* tussen CNS en *S. aureus*. Verder hebben andere studies ook onbekende SCC*mec* typen gevonden, of SCC elementen zonder *mec*A, welke een reservoir kunnen zijn voor antibiotica resistentie genen, in *S. aureus*. Tevens dient de rol van huisdieren en landbouwdieren te worden

onderzocht als mogelijke bron voor het SCC*mec*. Ontstaat het SCC*mec* in dieren in MRCNS of MRSA en wordt vervolgens de mens besmet, of zijn mensen de bron van CNS en *S. aureus* stammen welke SCC*mec* bevatten?

Er wordt gesuggereerd dat MRSA meer pathogeen is dan MSSA, maar weinig studies zijn uitgevoerd om dit te bewijzen. CA-MRSA, welke het SCC*mec* type IV en het PVL bevat, kan ernstige huidinfecties bij jonge volwassenen veroorzaken. Een relatie tussen resistentie en virulentie kan niet worden uitgesloten, omdat lage concentraties antibiotica de expressie van virulentiefactoren kunnen beïnvloeden.

Een andere vraag is waarom een groot percentage MRSA stammen resistent is voor ciprofloxacine. Het mechanisme van resistentie van fluoroquinolonen enerzijds en methicilline anderzijds is geheel verschillend. Fluoroquinolonen resistentie wordt hoofdzakelijk veroorzaakt door mutaties in het chromosoom, terwijl methicilline resistentie wordt veroorzaakt door SCC*mec*. Een mogelijkheid is dat MRSA stammen worden geselecteerd tijdens antibioticatherapie en dat antibioticadruk de verspreiding van multi-resistente *S. aureus* stammen stimuleert.

Duidelijk is dat onderzoek naar verspreiding van antibioticaresistente bacteriën een belangrijk onderwerp is voor verdere studies.

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Ruud

CURRICULUM VITAE

Ruud Deurenberg werd op 15 mei 1970 geboren in Heerlen, Nederland. Van 1986 tot 1991 studeerde hij chemie aan het Middelbaar Laboratorium Onderwijs (MLO) te Sittard. Na afloop besloot hij verder te studeren aan het Hoger Laboratorium Onderwijs (HLO), alwaar hij biochemie studeerde. Na voltooiing van zijn stage- en afstudeeropdracht bij de afdeling Medische Microbiologie van het academisch ziekenhuis Maastricht (azM) in 1995, werkte hij van 1995 tot 1998 als research analist bij deze afdeling. Van oktober 1998 tot juni 2000 was hij werkzaam als hoofdanalist bij de afdeling Biotechnologie van de Stichting Sanquin te Amsterdam. In juli 2000 begon hij te werken als research analist en laboratorium manager bij de afdeling Radiotherapie van het azM en sinds juni 2003 is hij werkzaam als assistent in opleiding (AIO) bij de afdeling Medische Microbiologie van het azM alwaar hij zijn promotieonderzoek verrichtte.

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8. **R.H. Deurenberg**, C. Vink, J. Hoogkamp-Korstanje and E.E. Stobberingh. Is ciprofloxacin resistance in *Staphylococcus aureus* a biological marker for methicillin-resistant *Staphylococcus aureus* in The Netherlands? Wetenschappelijke Voorjaarsvergadering NVMM/NVVM 2005, Arnhem, The Netherlands. 11 to 13 April 2005.
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Finding the treasures that lay hidden in my lonely cave...

(Kayak – Merlin)